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# FACTORS AFFECTING PORPHYRIN BIOSYNTHESIS

by

GERALD HERMAN HIRSCH

A THESIS

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ABSTRACT

A group of disorders known as porphyrias have been described which involve inborn or acquired derangements of the enzymes involved in heme biosynthesis. The over-production of porphyrins and porphyrin precursors in the livers of animals fed porphyria-inducing drugs results from an enhanced synthesis of the first enzyme of the porphyrin biosynthetic pathway viz., δ-aminolevulinic acid synthetase. Recently the porphyria-inducing activity of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine was investigated and it was shown that replacement of the 4-methyl group with a hydrogen atom resulted in a marked loss of activity. It was suggested that the 4-methyl substituent causes a twisting of the ethoxycarbonyl substituents out of the plane of the ring and that this molecular configuration was responsible for porphyria-inducing activity. It was the objective of Part I of this thesis to check this hypothesis. For this reason the porphyria-inducing activity of 3,5-diethoxycarbonyl-4-methylpyridine and 3,5-diethoxycarbonylpyridine was tested using an in vitro tissue culture method developed by Granick. The inactivity of these two planar pyridines provided evidence for the hypothesis suggested above. In further studies 3-ethoxycarbonyl-2,4,6-trimethylpyridine, which has only one ethoxycarbonyl substituent out of the plane of the ring, was found to be active, although less so than 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine which has two ethoxycarbonyl substituents out of the plane. Ethyl 2,4,6-trimethylbenzoate and diethyl 2,3,5,6-tetramethylterephthalate were also tested for porphyria-inducing activity. The former had an activity similar to that of 3-ethoxycarbonyl-2,4,6-trimethylpyridine while diethyl 2,3,5,6-tetramethylterephthalate had an

activity comparable to that of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine. These observations show that the essential feature in these porphyria-inducing compounds is a sterically hindered ethoxycarbonyl substituent and that the pyridine ring can be replaced by a benzene ring.

The emphasis placed by previous investigators on the importance of a free allyl group for porphyria-inducing activity is difficult to reconcile with the high activity of the pyridine compounds which do not contain an allyl group. Therefore some of the barbiturate group of compounds previously tested in whole animals have been re-investigated using chick embryo liver cells to evaluate the importance of a free allyl group for activity. A comparison of the porphyria-inducing activities of 2-allyl-2-isopropylacetamide and 2-propyl-2-isopropylacetamide is of interest since the only structural difference between these two compounds is the replacement of the allyl group by a propyl group. The activity of these compounds was similar in the <u>in vitro</u> system whereas in the whole animal 2-allyl-2-isopropylacetamide was reported to be active and 2-propyl-2-isopropylacetamide devoid of activity. Thus the free allyl group does not appear to be required in the <u>in vitro</u> system.

Because of the high activity found in the apparently chemically unrelated pyridine and 2-allyl-2-isopropylacetamide series of compounds, in Part Ic of this thesis we explored their possible common mechanism of action. On replacing the ethoxycarbonyl substituent of ethyl 2,4,6-trimethylbenzoate with an amide group, and the amide group of 2-allyl-2-isopropylacetamide with an ester group, we found that these new compounds had similar porphyria-inducing activity. Granick has suggested

that the critical feature in Griseofulvin that is important for porphyrin induction is the distance between the keto oxygen atoms. In Part Id of this thesis we have tested the porphyria-inducing activity of Griseofulvin and various analogues, and our results support the hypothesis of Granick.

Since the over-production of porphyrins is probably the result of an enhanced synthesis of δ-aminolevulinic acid synthetase, the possibility of inhibiting this enzyme was studied in Part II of this thesis. Commercial avidin, a protein obtained from egg white, has been found by Tait to inhibit δ-aminolevulinic acid synthetase of the photosynthetic organism Rhodopseudomonas spheroides. We have confirmed this observation, and using dialysis and column chromatography we have determined the approximate molecular weight of this inhibitor. However, further studies showed that commercial avidin did not inhibit the δ-aminolevulinic acid synthetase of chicken erythrocytes and chick embryo liver cells. These observations indicate that commercial avidin probably would be of little value in the treatment of porphyria.

ACKNOWLEDGEMENTS

The author wishes to express appreciation and gratitude to G. S. Marks, D. Phil., for his kindly counsel, his constant encouragement and his able guidance, all of which have contributed so much to the completion of this thesis. A sincere thanks is also due to Mr. G. L. Bubbar who synthesized some of the compounds used in this work.

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# INTRODUCTION

Most porphyrins are metabolic by-products originating in the course of heme biosynthesis and are widely distributed in nature. Porphyrins occur in the free form in small amounts and have the property of combining very readily with a variety of metals to form metalloporphyrins. The most important metalloporphyrins are the iron complexes or hemes. The erythropoietic system and the liver are important sites of porphyrin and heme formation. Heme formed in the erythropoietic system is utilized for hemoglobin formation while heme formed in the liver is incorporated into the enzymes cytochrome, peroxidase and liver catalase. Inborn and acquired derangements of enzymes involved in heme biosynthesis have been described and these disorders are designated porphyrias. These derangements result in an increased excretion of porphyrins or of their precursors, or both, in the excreta.

While early work on disorders of porphyrins metabolism was hampered by a lack of knowledge of normal porphyrin metabolism, the recent clarification of the steps in the biosynthesis of heme due to the brilliant work of Shemin[1] has facilitated studies in this field. Thus it has been shown[2] that the first step of porphyrin biosynthesis involves the condensation of glycine (II) and succinyl CoA (I) to δ-aminolevulinic acid (III) by the enzyme δ-aminolevulinic acid synthetase. δ-Aminolevulinic acid dehydrase catalyses the condensation of two molecules of δ-aminolevulinic acid to yield the monopyrrole porphobilinogen (IV). Under the influence of two enzymes[3], uroporphyrinogen synthetase and uroporphyrinogen isomerase, four molecules of porphobilinogen are condensed to form the macrocycle, uroporphyrinogen III (V). Uroporphyrinogen decarboxylase[4] is responsible

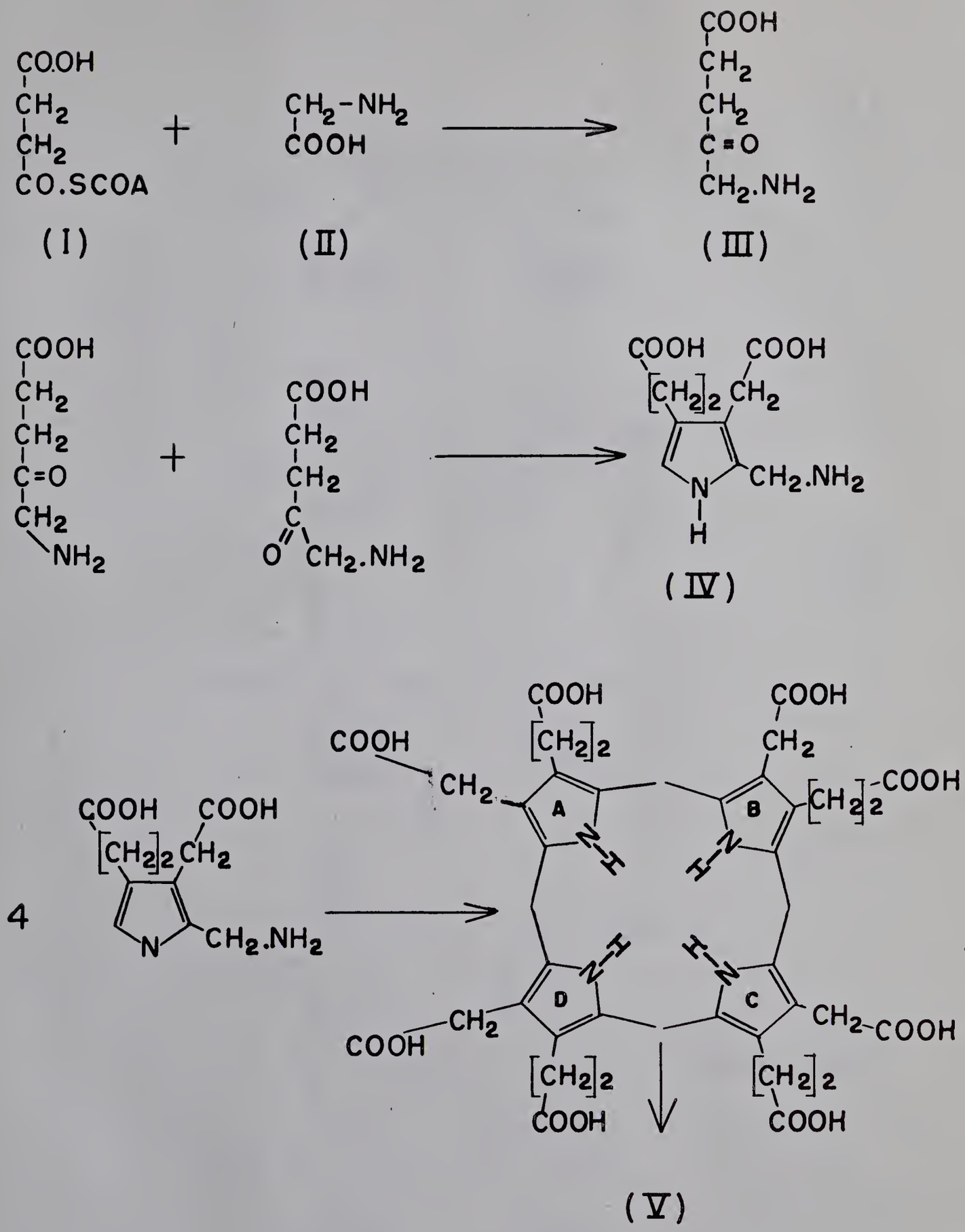
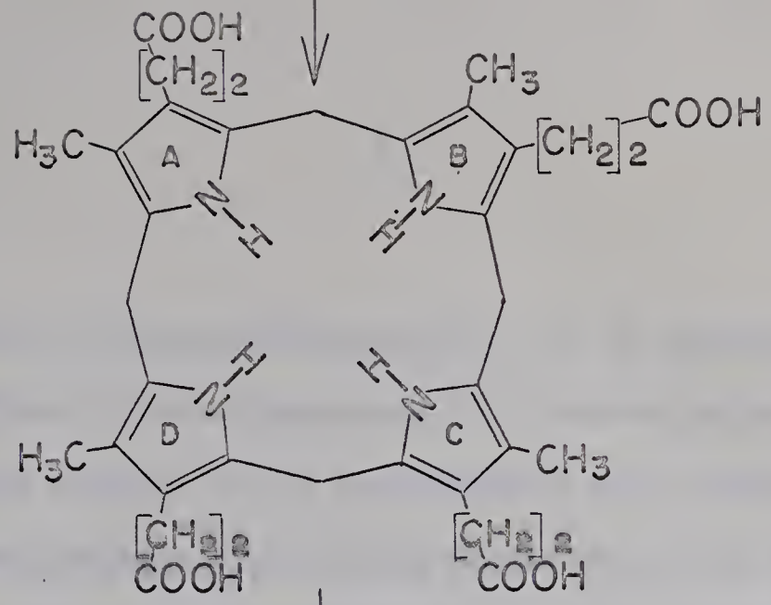



Figure 1. Biosynthesis of Heme

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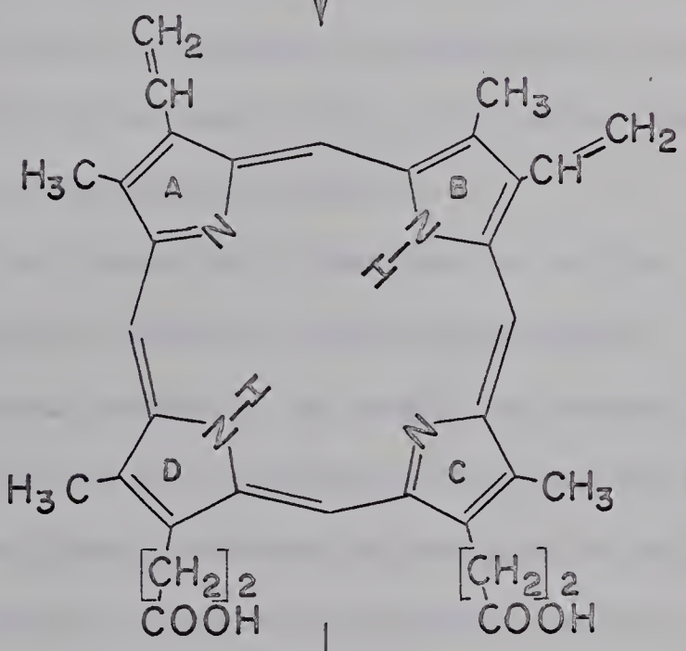


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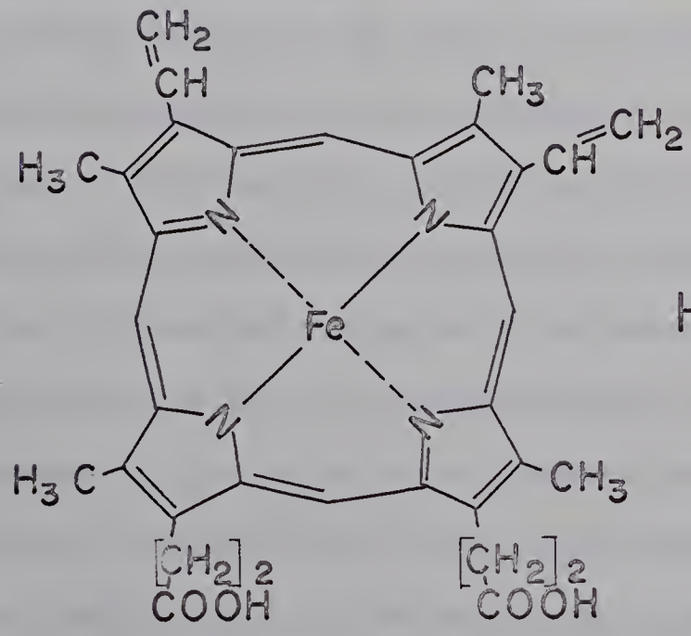



Heme (VIII)

Figure 1: Biosynthesis of Heme, cont'd

for the production of coproporphyrinogen III (VI) by decarboxylation of the acetic acid residues of uroporphyrinogen III. Oxidation, by the enzyme coproporphyrinogen oxidase, of the two propionic acid groups in rings A and B of coproporphyrinogen III to vinyl groups and of the methylene bridges to methine bridges, affords protoporphyrin IX (VII). The final step in the biosynthesis of heme is the enzymic incorporation of ferrous iron into protoporphyrin IX to give heme (VIII). The various steps in this biosynthetic pathway are depicted in figure 1.

The key to the present day classification of the porphyrias was provided by the studies of Schmid, Schwartz and Watson.[5] These studies revealed that in some cases of porphyria the porphyrins accumulated in the bone marrow, particularly in the normoblastic nucleus, while in other cases, porphyrins and porphyrin precursors accumulated in the liver. For this reason the disorders of porphyrin metabolism have been classified as erythropoietic and hepatic porphyria. The erythropoietic and hepatic porphyrias have been subdivided on the basis of the clinical features observed, the mode of inheritance and the porphyrins or porphyrin precursors produced. Two types of erythropoietic porphyria have been recognized viz., congenital erythropoietic porphyria and congenital erythropoietic protoporphyria.[5] In the erythropoietic porphyrias the metabolic defect is limited to the red cells and all of the abnormalities which appear in these patients are secondary to this defect in erythrocyte metabolism. The hepatic porphyrias are subdivided into three major types viz., acute intermittent porphyria (Swedish type), cutaneous hepatic porphyria (South African type), and acquired hepatic porphyria.[6] In the hepatic porphyrias the

defect appears to be limited to liver cells.

Acute intermittent porphyria is the most important member of the group of porphyria diseases and is clinically distinguishable from the others by the dominance of gastrointestinal and neurologic symptoms and the absence of skin photosensitivity.[6] In the acute and latent phases of this disease, and often in states of remission, patients excrete in the urine excessive quantities of the porphyrin precursors porphobilinogen and δ-aminolevulinic acid. Among the factors believed responsible for precipitating the clinical manifestations of the disease, the administration of various drugs and various stress conditions are most important.

Unlike acute intermittent porphyria, photosensitivity is one of the major manifestations of cutaneous hepatic porphyria. In addition, acute attacks, neurologic involvement, and induction by drugs are features associated with this disease. The prominent biochemical finding is the high fecal excretion of coproporphyrin and protoporphyrin.[7]

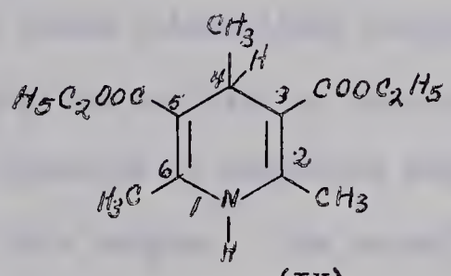
There is an acquired form of hepatic porphyria which clinically resembles cutaneous hepatic porphyria but is non-hereditary.[8] In 1956 a large number of Turks consumed wheat intended for planting purposes which was treated with the fungicide hexachlorobenzene, and acquired a hepatic form of porphyria. Photosensitivity, as shown by severe scarring of the hands and face, was the predominant clinical manifestation and this was accompanied by the excretion of uro- and copro-porphyrin in the urine. The etiology of this disease was first suggested by Cam[9] and this suggestion was supported by the finding that hexachlorobenzene readily induced a porphyria when fed to rats[10], guinea pigs, mice and rabbits.[11] The

porphyria observed in Turkey provides evidence for the occurrence in man of a purely acquired and not genetically predetermined form of hepatic porphyria.

Since the introduction of barbiturates into clinical medicine, evidence has accumulated that relatively small amounts of these drugs may precipitate acute, and at times, fatal attacks in clinically latent porphyria.[12] Recently a variety of drugs have been demonstrated to act in a similar fashion to the barbiturates; these include griseofulvin, meprobamate (Miltown) chloradiazepoxide (Librium) and 5,5-diphenylhydantoin (Dilantin).[13] While small doses of these drugs can induce an attack of porphyria in patients carrying the inherited trait of acute intermittent porphyria or cutaneous hepatic porphyria, much larger doses of these drugs will cause a disordered porphyrin metabolism in normal animals.[6, 14] The disorders of porphyrin metabolism induced in normal animals by drug administration are referred to as experimental porphyrias. Although there is a striking similarity in the biochemical abnormality observed in the experimental and in the hepatic porphyrias, the clinical manifestations observed in the hepatic porphyrias are not reproduced in the experimental porphyrias. For this reason caution is required in extrapolating the data from the experimental animal porphyrias to the inherited disease. Despite this limitation however, valuable information regarding the inherited disease has been obtained from studies of the experimental porphyrias.

With the steps of porphyrin biosynthesis clarified it was possible for Granick and Urata[15, 16] to study the metabolic defect occurring in experimental porphyria. They were able to produce a disorder of

porphyrin metabolism in the livers of guinea pigs, resembling that seen in acute intermittent porphyria, by the administration of the porphyrinogenic compound 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl-pyridine (DDC,IX).



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The level of the liver enzymes involved in porphyrin biosynthesis was found to be normal with the exception of the first enzyme of the pathway viz., δ-aminolevulinic acid synthetase.[17] Whereas in the normal guinea pig liver this enzyme could barely be detected, its level in the porphyric guinea pig was elevated forty-fold.[17] It thus appeared that porphyrin biosynthesis was normally controlled by the level of δ-aminolevulinic acid synthetase and that the overproduction of porphyrins and porphyrin precursors in porphyria was the result of an increased activity of this enzyme. Granick, using a tissue culture technique which he developed, cultured chick embryo liver parenchyma cells in the presence of DDC and found an increased activity of δ-aminolevulinic acid synthetase.[18] However after the addition of Mitomycin C, Actinomycin D and other inhibitors of protein synthesis, DDC did not cause any increase in the activity of δ-aminolevulinic acid synthetase.[18] It was thus apparent that the mode of action of DDC on normal cells capable of carrying out protein synthesis was by direct stimulation of the synthesis of δ-aminolevulinic acid synthetase.

On the basis of these experiments with drug-induced porphyrias, Granick[8] suggests that there is a mechanism for the control of this enzyme by repression of its formation and that these drugs interfere with the repressor control mechanism. He has further suggested that in the hereditary disease of acute intermittent porphyria the regulatory control mechanism governing the activity of the structural gene for δ-aminolevulinic acid synthetase is defective and does not sufficiently repress the formation of this enzyme.[8] The recent demonstration of an increased hepatic level of δ-aminolevulinic acid synthetase in a patient with acute intermittent porphyria is in accord with this suggestion.[19]

An important question that remains to be answered concerns the nature of the cellular receptor with which the porphyria-inducing drugs interact to interfere with the repressor control mechanism. In the mechanism controlling enzyme formation proposed by Jacob, Monod, and Wollman,[20] a specific repressor molecule is considered to inhibit a specific DNA region (operon) from being decoded into mRNA, thus preventing the synthesis of an enzyme. Granick[21] considers the aporepressor for δ-aminolevulinic acid to be a protein to which is attached a corepressor which is heme and that the aporepressor and corepressor combine to form an active repressor. He further suggests that a porphyria-inducing compound competes with and displaces heme from the corepressor site resulting in an inactive repressor so that more δ-aminolevulinic acid can be made.[21] Figure 2 pictures a hypothetical corepressor site which illustrates how this site might be occupied by DDC as envisaged by Granick.

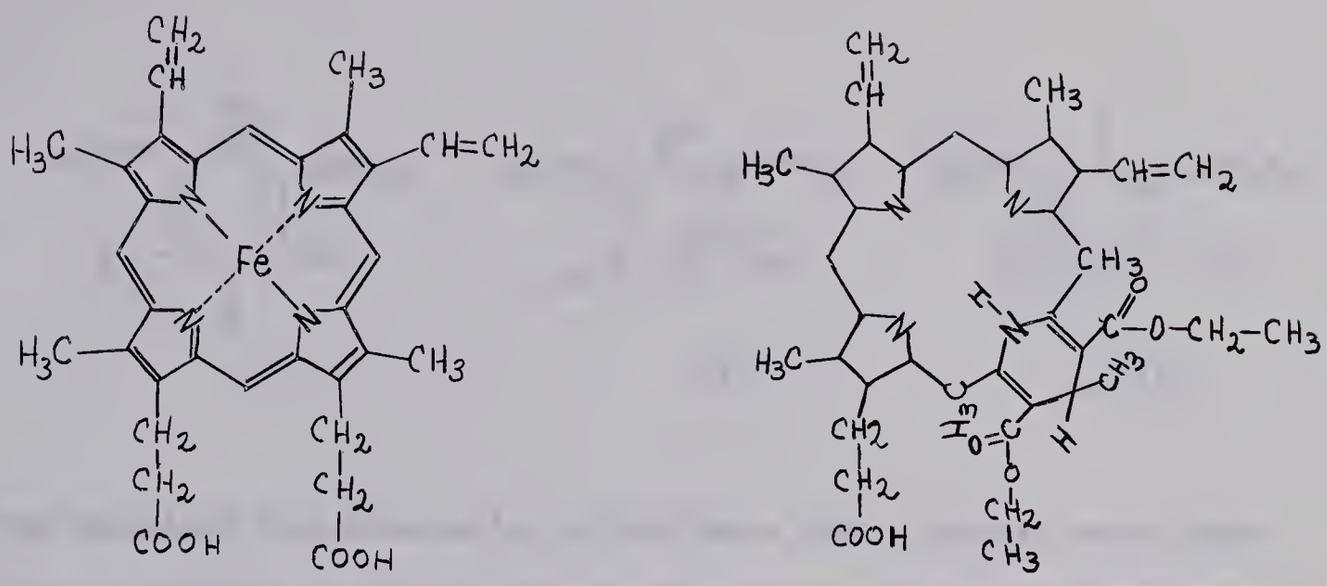
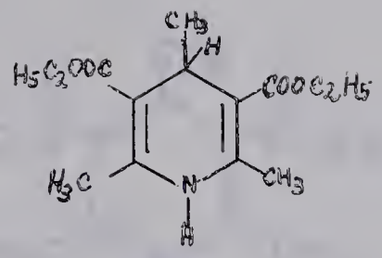



Figure 2. Occupation of a Hypothetical Heme Corepressor Site by DDC

Studies of the molecular nature of drug receptors are based on the assumption that small molecules whose biological action is specific and structure-dependent have a molecular structure complementary to the site at which they act.[22] A study of the structure-activity relationship of porphyria-inducing drugs is therefore a means with which the nature of the drug-receptor may be explored and the hypothesis of Granick analyzed. In Part I of this thesis studies investigating this subject are described.

In recent years Marks et al.[23] investigated the porphyria-inducing activity of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, IX) and the corresponding pyridine (X). These workers showed that the 4-methyl substituent could be replaced by other alkyl substituents with retention of activity, but that replacement of this methyl group with a hydrogen atom as in pyridine (XI) caused a marked loss of activity.



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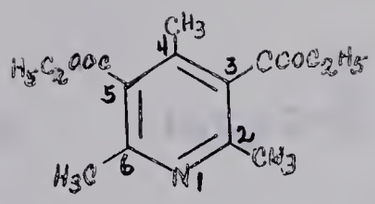

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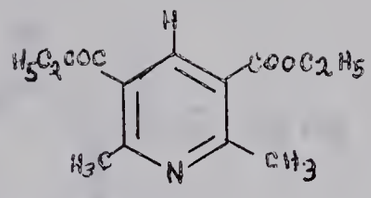
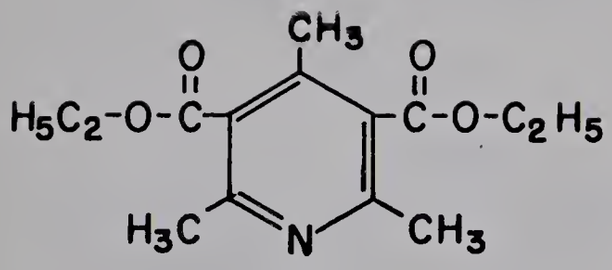


(XI)

They explained this observation on the basis that a 4-alkyl substituent causes a twisting of the ethoxycarbonyl substituents out of the plane of the ring. It was suggested that this non-planar relationship between the ethoxycarbonyl substituents and the pyridine ring was required for optimal porphyria-inducing activity. The non-planarity of the pyridine (X) and the planar character of the pyridine (XI) is clearly shown in the Fisher-Taylor-Hirschfelder models in figure 3. Marks et al.[23] suggested that if this hypothesis were correct then removal of the 2- and 6-methyl substituents of pyridine (X), yielding pyridine (XII), would result in coplanarity of the ethoxycarbonyl substituents with the ring and consequently in reduced activity. Furthermore, if this hypothesis is correct then 3,5-diethoxycarbonylpyridine (XIII), 2,4,6-trimethylpyridine (XIV), and 3-ethoxycarbonyl-2,4,6-trimethylpyridine (XV) should be inactive. This follows from the fact that pyridines (XIII) and (XIV) are planar and pyridine (XV) has only one ethoxycarbonyl substituent out of the plane of the ring. In Part Ia of this thesis the porphyria-inducing activity of pyridine (X) has been compared with that of pyridines (XII), (XIII), (XIV) and (XV) in chick embryo liver cells in order to investigate the validity of the hypothesis of Marks et al.

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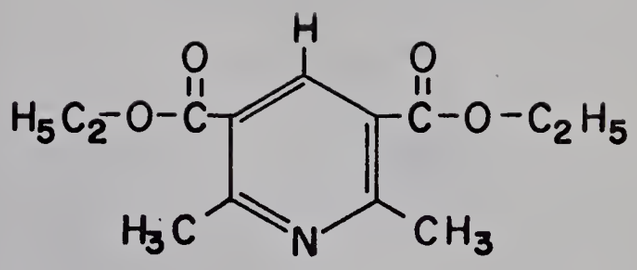


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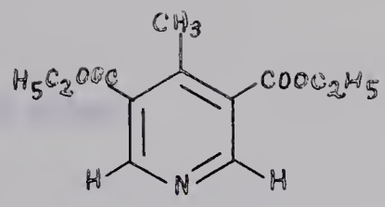
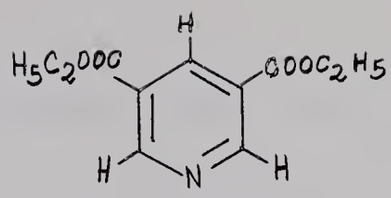
3,5- DIETHOXYCARBONYL
-2,4,6-TRIMETHYLPYRIDINE

3,5- DIETHOXYCARBONYL
-2,6- DIMETHYLPYRIDINE

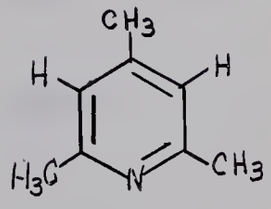
Figure 3



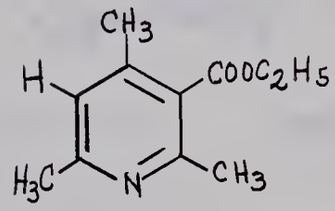
(XII)



(XIII)



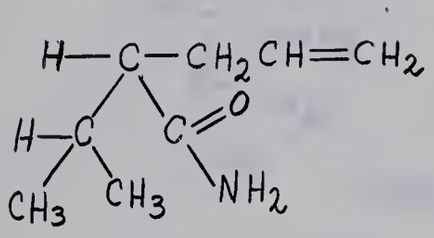
(XIV)



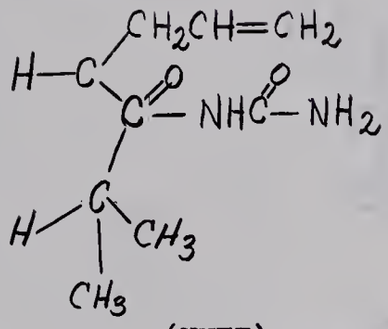
(XV)

As mentioned earlier, small amounts of some of the barbiturates can precipitate acute, and at times, fatal attacks of clinically latent porphyria. Goldberg and Rimington[6] have investigated the relationship between chemical structure and porphyria-inducing activity in this group of compounds. They concluded that the structural features required for porphyria-inducing activity in rabbits were one allyl group together with an acid amide as in 2-allyl-2-isopropylacetamide (XVI), or a ureide as in 2-isopropylpent-4-enoylurea (XVII) or a cyclic ureide as in 5,5-diallylbarbituric acid (XVIII). These results were confirmed by Stich and Decker[24] and by Talman, Labbe and Aldrich.[25] It is difficult to reconcile the high degree of structural specificity for porphyria-inducing activity found in the barbiturate group of drugs with the activity exhibited by the apparently unrelated pyridine and dihydropyridine compounds. In particular the emphasis placed by these investigators[6,24,25] on the importance of a free allyl group for porphyria-inducing activity in whole animals is difficult to reconcile with the high activity of the pyridine group of compounds which do not contain

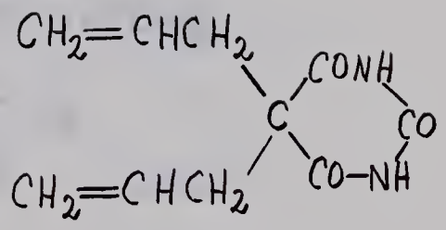
an allyl group.



(XVI)



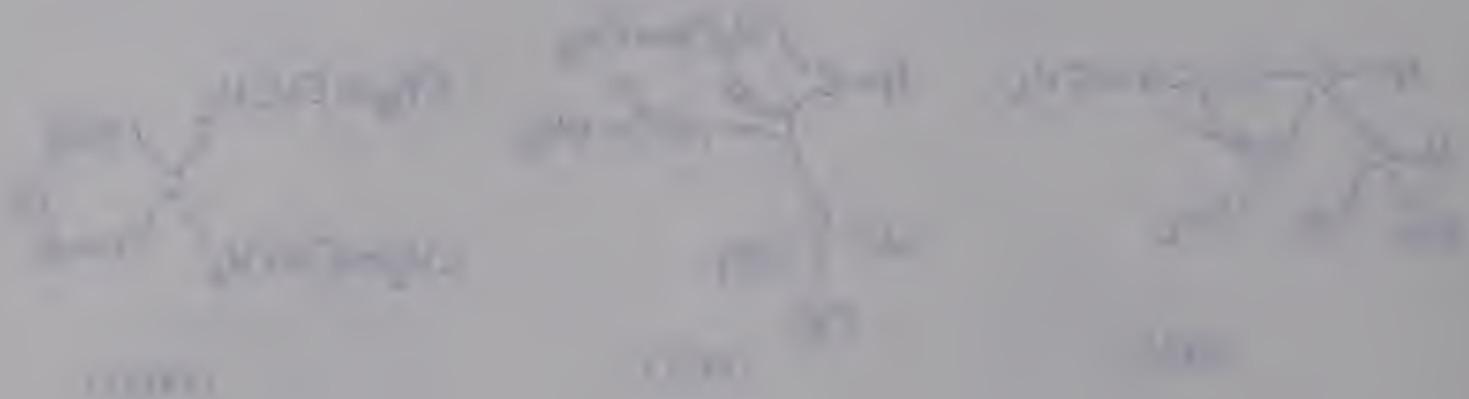
(XVII)



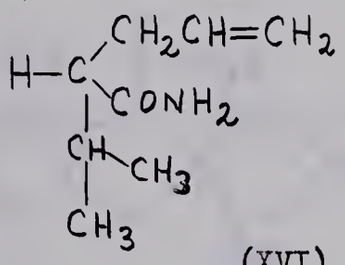
(XVIII)

The action of a drug in an animal is the result of a complex series of processes which may require specific features in the drug molecule. Studies in whole animals are therefore unsuited for the interpretation of pharmacological action on a molecular level. The in vitro chick embryo liver cell system is suitable for this purpose and for this reason the importance of the free allyl group has been re-investigated in this system. Thus the activity of 2-allyl-2-isopropylacetamide (XVI) has been compared with that of 2-propyl-2-isopropylacetamide (XIX) and the activity of 2-isopropylpent-4-enoylurea (XVII) with that of 2-propylpentanoylurea (XX), and the results of this study are reported in Part Ib of this thesis.

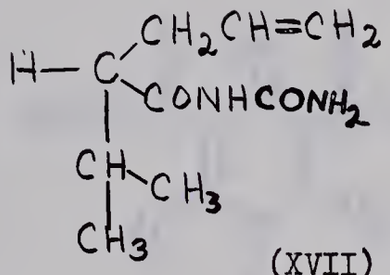
Talman et al.[25] compared the porphyria-inducing activities of a variety of drugs related to the barbiturates by injecting them into the yolk sac of eight day old chick embryos and measuring the porphyrin concentration of the allantoic fluid. It was of interest to compare the activities reported for some of these compounds by Talman et al. with



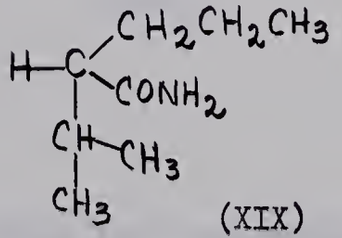
their activity in the chick embryo liver cell system. These results are also reported in Part Ib of this thesis.



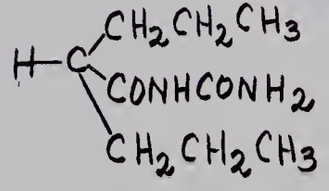
(XVI)



(XVII)

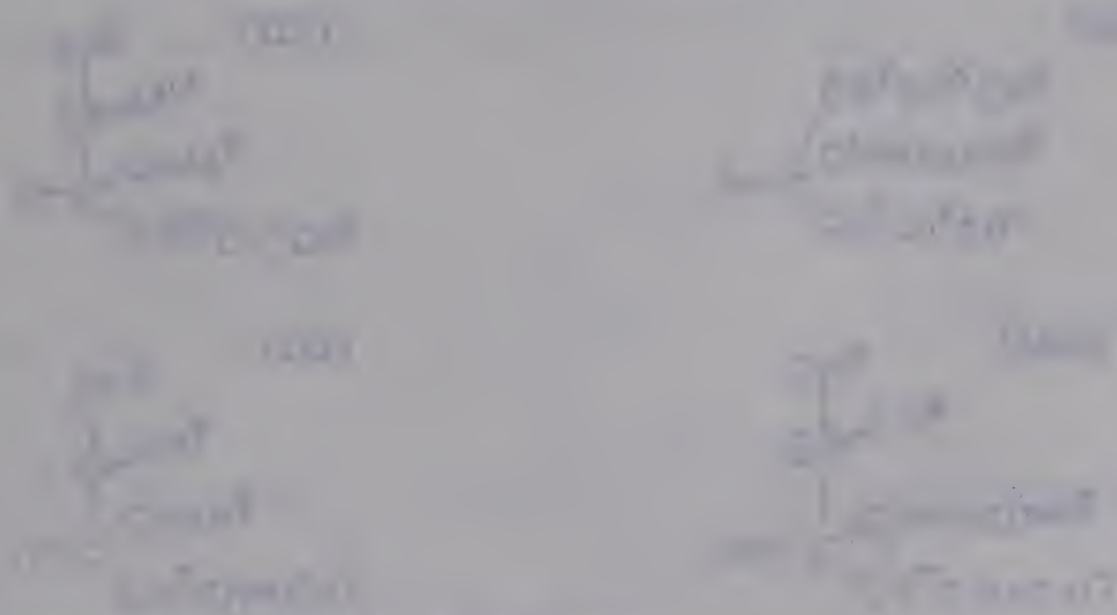


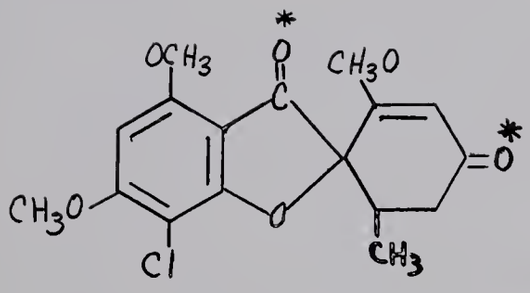
(XIX)



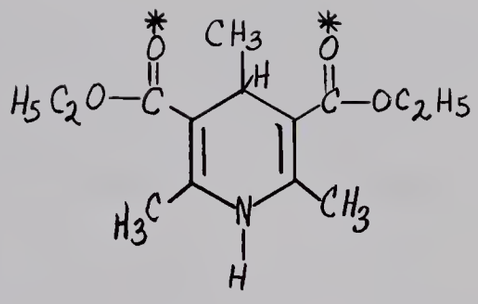
(XX)

A number of investigators have established that Griseofulvin (XXI), an antifungal agent, is an active porphyria-inducing compound.[13,21] Granick[21] has suggested that the critical feature in the Griseofulvin molecule for porphyria-inducing activity is the distance between the keto oxygen atoms (starred in XXI). Granick has found that it is possible to twist a Courtald model of DDC so that the distance between the keto oxygen atoms of DDC (starred in IX) is the same as that in Griseofulvin.[21] To investigate this hypothesis, and in an effort to determine the functional groups required in Griseofulvin for porphyria-inducing activity, Griseofulvin and various analogues have been tested in the chick embryo liver cell system. These results are presented in Part Id of this thesis.



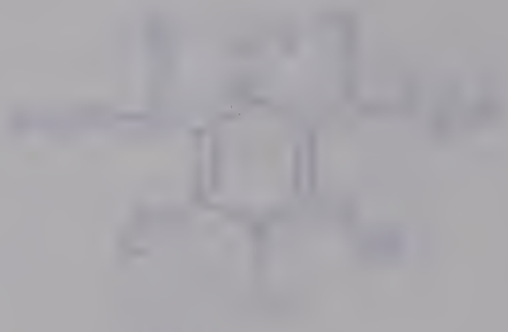


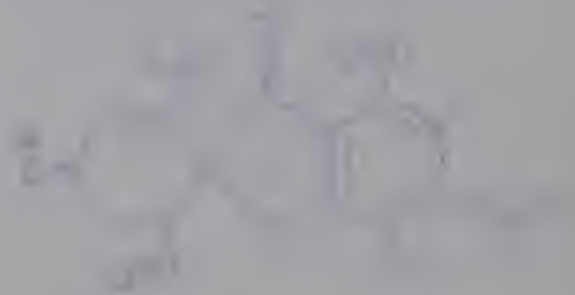
(XXI)



(IX)

Since the over-production of porphyrins in the various forms of porphyria is probably the result of an enhanced synthesis of δ-aminolevulinic acid synthetase, it was of interest to study the possibility of inhibiting this enzyme. If an inhibitor could be found, it is possible that it might be of value in relieving some of the symptoms found in the various forms of porphyria. Tait[26] has shown that commercial avidin, a protein obtained from egg white, contains a component which inhibits the δ-aminolevulinic acid synthetase of the photosynthetic organism Rhodopseudomonas spheroides, and it was thought that this inhibitor might be of value in human porphyria. For this reason studies were carried out to separate this inhibitor from avidin and to determine its approximate molecular weight. Furthermore, the ability of this inhibitor to inhibit δ-aminolevulinic acid synthetase in chicken erythrocytes and chick embryo liver cells was studied. These studies are reported in Part II of this thesis.



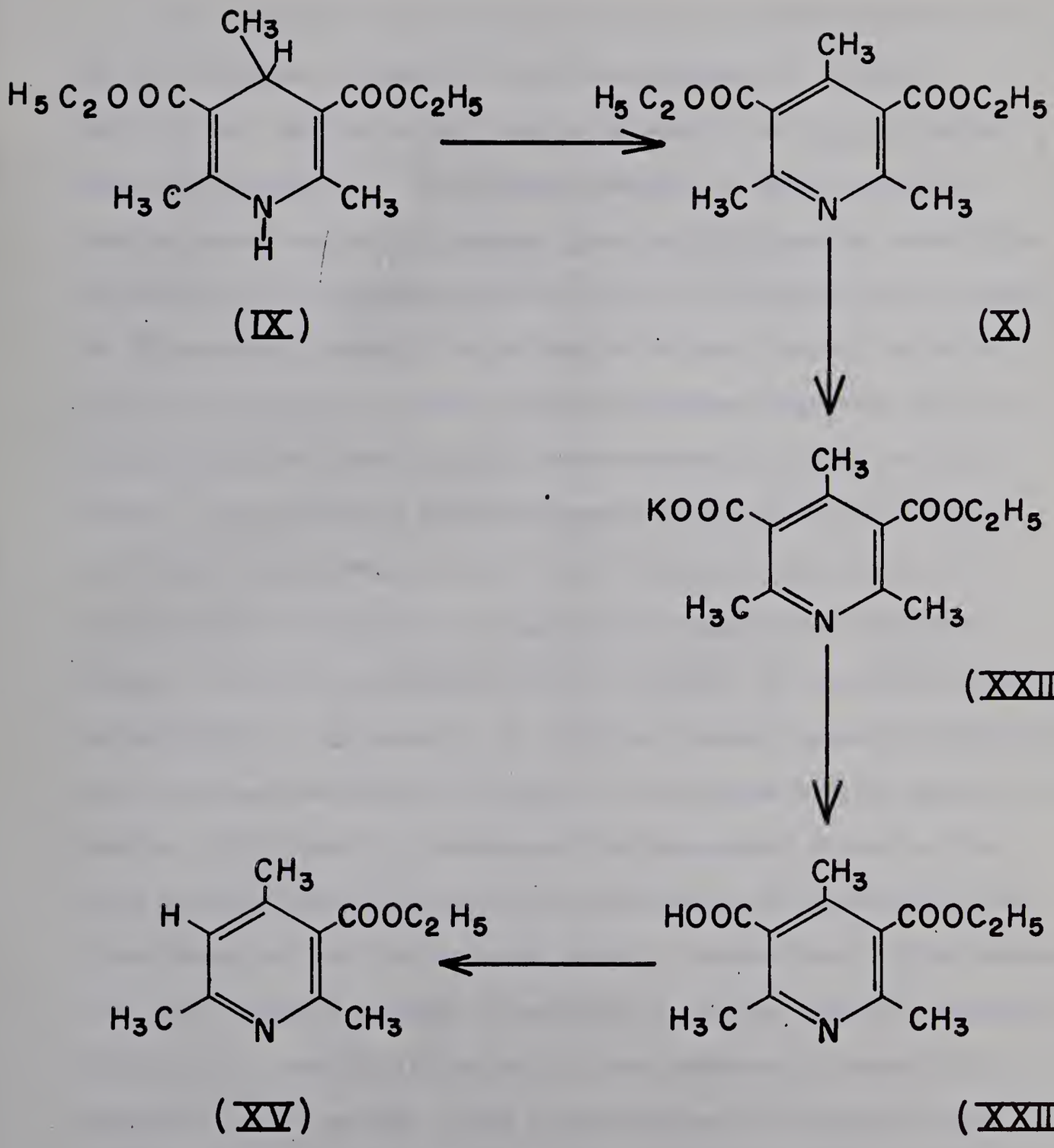


MAIN SECTION

## PART Ia

As mentioned in the introduction, the purpose of Part Ia of this thesis was to compare the porphyria-inducing activity of pyridine (X) with that of pyridines (XII), (XIII), (XIV) and (XV) in chick embryo liver cells. 2,4,6-Trimethylpryidine (XIV) was obtained from the Aldrich Chemical Company and pyridines (XII) and (XIII) were obtained from Marks and Bubbar (Department of Pharmacology, University of Alberta). The identity of pyridine (XIII) was confirmed by the above two workers by a comparison of the melting point (50-50.5°C) with that reported in the literature (51°C).[27] The identity of pyridine (XII) was confirmed by its nuclear magnetic resonance spectrum and by analysis of its picrate.

Pyridines (X) and (XV) were not available and were therefore synthesized by the following route: The starting point for the synthesis was 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (IX, Eastman Kodak Company) which was converted into pyridine (X) by oxidation with chloranil. Treatment of the latter compound with potassium hydroxide in absolute alcohol yielded the pyridine (XXII), which upon treatment with hydrochloric acid afforded the pyridine acid (XXIII). Upon heating at 250°C the pyridine acid (XXIII) was decarboxylated to yield the desired pyridine ester (XV).


CH3
H
H5C2OOC
COOC2H5
H3C
N
CH3
H
(IX)
CH3
H5C2OOC
COOC2H5
H3C
N
CH3
(X)
CH3
KOOC
COOC2H5
H3C
N
CH3
(XXII)
CH3
HOOC
COOC2H5
H3C
N
CH3
(XXIII)
CH3
H
COOC2H5
H3C
N
CH3
(XV)

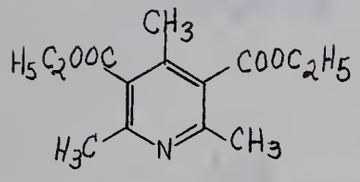
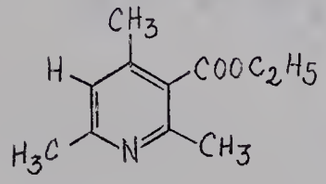
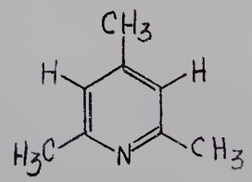
The ability of 3,5-diethoxycarbonyl-2,4,6-trimethylpryidine (X) and its analogues to induce an increased synthesis of δ-amino-levulinic acid synthetase was studied by means of an in vitro method developed by Granick.[21] This method consists of adding porphyria-inducing chemicals to chick embryo liver cells cultured on cover slips and measuring the porphyrin formed by means of fluorescence microscopy. The fluorescence intensity was scored as follows: +4, all colonies fluoresce intensely; +3, most colonies fluoresce intensely; +2, most colonies fluoresce partially; +1, some colonies fluoresce partially. Granick[16] has shown that the fluorescence values of +4,+3,+2,+1 are equivalent, respectively, to 0.5 to 1.0, 0.25 to 0.5, 0.12 to 0.25, and less than 0.12 mμ moles of coproporphyrinogen per milligram of protein. In order to eliminate bias in scoring, the samples were evaluated independently by two persons. In addition, several compounds which had previously been evaluated by Granick in this system were included in our studies. The similarity between our two independent scores and the score given by Granick indicated that the method was reproducible and strengthened our confidence in the method. Moreover most of the compounds have been tested in a series of experiments and the degree of reproducibility of the results, as indicated in the experimental section, is excellent. All compounds tested were dissolved in absolute alcohol and one to five microlitres of the alcoholic solution was added to one millilitre of culture medium. In control tests it was found that the addition of one to five microlitres of absolute alcohol did not induce any fluorescence in the cells.

Occasionally high concentrations of compounds inhibited growth or killed the cells. Inhibition of growth or injury of the cells, when they occurred, could be recognized by retraction of the smooth filmy edge of the cell colonies when viewed with phase optics. Death of the parenchymal cells was indicated by detachment of the cells from the cover slip. The results obtained by means of this technique are recorded in Table 1. The values of the fluorescence intensity recorded represent the average values obtained in at least two experiments in which each drug concentration was tested in duplicate or triplicate.

As indicated in the introduction, the importance of the 4-alkyl substituent for porphyria-inducing activity in pyridine (X) has been attributed by previous workers to the fact that it causes a twisting of the ethoxycarbonyl substituents out of the plane of the ring.[23] Moreover, it was suggested by these workers that if this hypothesis were correct then removal of the 2- and 6-methyl substituents of pyridine (X) yielding pyridine (XII) would result in co-planarity of the ethoxycarbonyl substituents with the ring and therefore in reduced activity. The finding (Table 1) that pyridine (XII) is devoid of activity therefore provides support for the hypothesis of Marks et al.[23] The inactivity of the planar pyridine (XIII) is in accord with the interpretation suggested by Marks et al. while the slight but definite activity exhibited by pyridine (XIV) was not anticipated and remains to be explained. The inactivity of pyridine (XXIII) was expected since it would exist as an anion at physiological pH and would not be expected to cross the cell membrane. The activity of pyridine (XV), which has only one ethoxy-

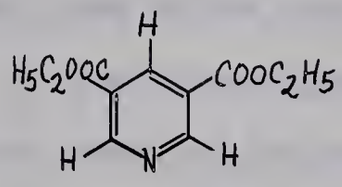
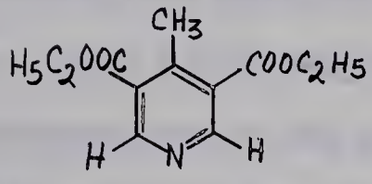
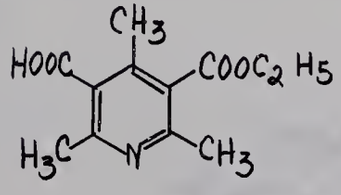
TABLE 1

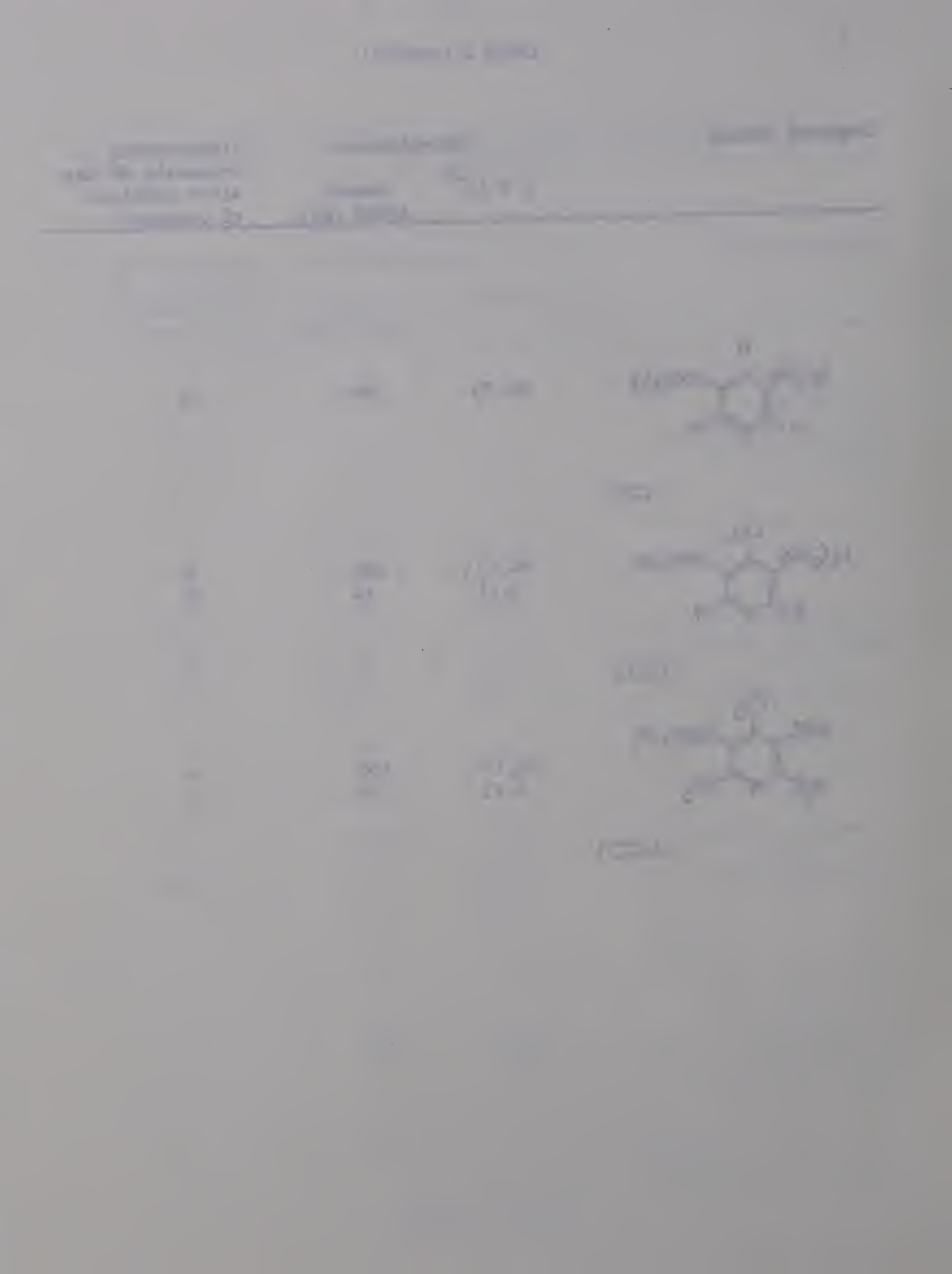
PYRIDINE DERIVATIVES THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
| no addition | | | 0 |
| absolute alcohol (5µl) | | | 0 |
|  (X) | 3.77 | 10 | +3 |
| | 2.26 | 6 | +2½ |
| | 1.13 | 3 | +2 |
| | .38 | 1 | +½ |
| | .076 | 0.2 | 0 |
|  (XV) | 51.74 | 100 | +3½ |
| | 5.17 | 10 | +1 |
| | 1.55 | 3 | trace |
| | .52 | 1 | 0 |
|  (XIV) | 82.52 | 100 | +1 |
| | 8.25 | 10 | 0 |

(Table 1 cont'd)

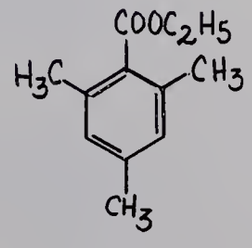
TABLE I (cont'd)

| Compound Tested | Concentration M x $10^{-5}$ | Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
|  (XIII) | 44.79 | 100 | 0 |
|  (XII) | 42.13<br>4.21 | 100<br>10 | 0<br>0 |
|  (XXIII) | 42.10<br>4.21 | 100<br>10 | 0<br>0 |

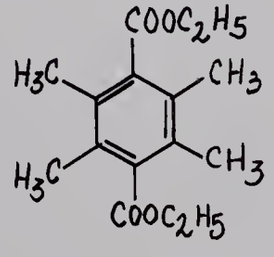


carbonyl substituent out of the plane, was less than that of pyridine (X) which has two ethoxycarbonyl substituents out of the plane of the ring. Nevertheless it has considerable activity (Table 1), indicating that the basic structural feature necessary for porphyria-inducing activity in these compounds is an ethoxycarbonyl substituent which is forced out of the plane of the ring.

It thus appeared possible that the pyridine and dihydropyridine rings are not essential for porphyria-inducing activity and that other ring systems might be able to replace them. Therefore two benzene derivatives (obtained from Marks and Bubbar) were tested for porphyria-inducing activity viz., ethyl 2,4,6-trimethylbenzoate (XXIV), which has one ethoxycarbonyl substituent out of the plane, and diethyl 2,3,5,6-tetramethylterephthalate (XXV), which has two ethoxycarbonyl substituents out of the plane of the ring.

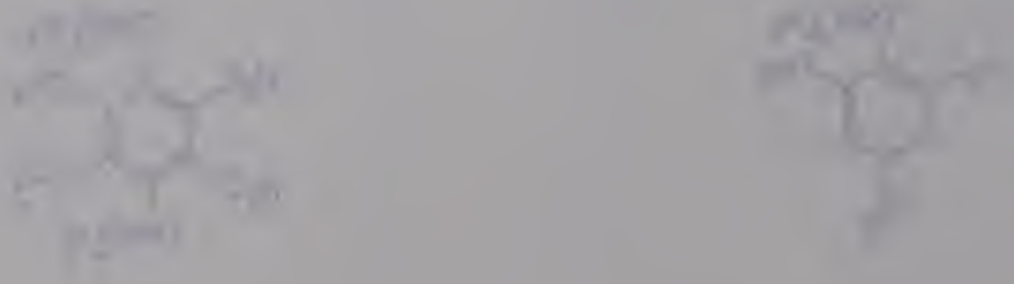



(XXIV)




(XXV)

The activity of ethyl 2,4,6-trimethylbenzoate (XXIV, Table 2) was found to be similar to that of the pyridine monoester 3-ethoxycarbonyl-2,4,6-trimethylpyridine (XV, Table 1) which also has one ethoxycarbonyl substituent out of the plane of the ring. These observations support the



hypothesis that the essential feature in these porphyria-inducing compounds is a sterically hindered ethoxycarbonyl substituent and show that the pyridine ring can be replaced by a benzene ring with no loss of activity. The addition of a second sterically hindered ethoxycarbonyl sutstituent, while not essential for porphyria-inducing activity, enhances the activity in both the pyridine and benzene compounds. The high activity of a variety of esters (XXVI, XXVII, XXVIII, Table 2) of tetramethylterephthalic acid demonstrate that the nature of the alcoholic moiety of the ester groups can be varied within considerable limits without any major change in activity. The inactivity of the free acids (XXIX, XXX) was expected since they would exist as anions at physiological pH and would not be expected to cross the cell membrane.

TABLE 2

BENZENE DERIVATIVES THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
| ethyl 2,4,6-trimethyl-benzoate (XXIV) | 52.0 | 100 | +2.5 |
| | 5.2 | 10 | +1 |
| diethyl 2,3,5,6-tetra-methylterephthlate (XXV) | 3.6 | 10 | +3.5 |
| | 1.1 | 3 | +2 |
| | 0.4 | 1 | +1 |
| dimethyl 2,3,5,6-tetra-methylterephthalate (XXVI) | 4.0 | 10 | +3.5 |
| | 0.4 | 1 | +1 |
| dipropyl 2,3,5,6-tetra-methylterephthalate (XXVII) | 3.3 | 10 | +4 |
| | 0.3 | 1 | +2 |
| diisopropyl 2,3,5,6-tetramethylterephthalate (XXVIII) | 3.3 | 10 | +3.5 |
| | 0.3 | 1 | +1.5 |
| tetramethylterephthalic acid (XXIX) | 45.0 | 100 | 0 |
| | 4.5 | 10 | 0 |
| 2,4,6-trimethylbenzoic acid (XXX) | 60.9 | 100 | 0 |
| | 6.1 | 10 | 0 |

## PART Ib

The emphasis placed by previous investigators[6] on the importance of a free allyl group in the barbiturate group of compounds for porphyria-inducing activity is difficult to reconcile with the high activity of the pyridine compounds which do not contain an allyl group. Therefore some of the barbiturate group of compounds previously tested in whole animals have been re-investigated using chick embryo liver cells to evaluate the importance of a free allyl group for activity. This *in vitro* system was used since it is more suitable for the interpretation of pharmacological action on a molecular level.

A comparison of the porphyria-inducing activities of 2-allyl-2-isopropylacetamide (XVI) and 2-propyl-2-isopropylacetamide (XIX) is of interest since the only structural difference between these two compounds is the replacement of the allyl group by a propyl group. The activity of these compounds is similar in the *in vitro* system (Table 3), whereas in the intact animal 2-allyl-2-isopropylacetamide (XVI) was reported to be active and 2-propyl-2-isopropylacetamide (XIX) devoid of activity.[6] Thus the free allyl group does not appear to be required for activity in the *in vitro* system, and this conclusion is supported by the comparable activities (Table 3) of 2-isopropylpent-4-enoylurea (XVII) and 2-propylpentanoylurea (XX). On the basis of these results it appears that all of these compounds have intrinsic activity, but that the free allyl group endows the molecule with suitable physico-chemical characteristics enabling it to achieve a significant concentration at the liver cell when administered to whole animals.

TABLE 3

ANALOGUES OF 2-ALLYL-2-ISOPROPYLACETAMIDE THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
| 2-allyl-2-isopropyl-acetamide (XVI) | 71.0 | 100 | +3 |
| | 14.2 | 20 | +2 |
| | 3.5 | 5 | +1 |
| | 0.7 | 1 | trace |
| 2-propyl-2-isopropyl-acetamide (XIX) | 70.0 | 100 | +3½ |
| | 14.0 | 20 | +2 |
| | 3.5 | 5 | +1 |
| | 0.7 | 1 | trace |
| 2-isopropylpent-4-enoylurea (XVII) | 54.3 | 100 | +2.5 |
| | 27.2 | 50 | +1 |
| | 2.7 | 5 | trace |
| 2-propylpentanoylurea (XX) | 54.0 | 100 | +2 |
| | 27.0 | 50 | +1 |
| | 2.7 | 5 | trace |

In 1957 Talman et al.[25] compared the porphyria-inducing activities of several barbiturate and related drugs by injecting them into the yolk sac of eight day old chick embryos, and measuring the porphyrin concentration of the allantoic fluid. It was of interest to compare the activities, reported for some of the compounds by Talman et al., with their activity in the chick embryo liver cell system. The results obtained by the two methods were found to be similar. Thus 2-allyl-2-isopropylacetamide, (XVI) 2-isopropylpent-4-enoylurea (XVII) and Seconal (XXXI) were active, 5,5-diallylbarbituric acid (XVIII) was weakly active, and Isoniazid (XXXII) and α-allylmalonamide (XXXIII) inactive (Table 4). An important difference was the finding that 2-propylpentanoylurea (XX) was active in the chick embryo liver cell system, but inactive when injected into the chick embryo.

The tissue culture method thus offers several important advantages over previous methods of evaluating porphyria-inducing activity:
(1) This method is useful in demonstrating porphyria-inducing activity in compounds thought to be inactive by means of <u>in vivo</u> experiments.
(2) A large number of compounds can be tested readily and the laborious analysis of porphyrins and porphyrin precursors in the excreta of whole animals is avoided. (3) Only small amounts of the compounds are required, in contrast to the large amounts needed in tests with whole animals.
(4) This method can be used to interpret pharmacological action on a molecular level. Studies of structure-activity relationships in the whole animal suffer from the fact that a distinction cannot be drawn between the effects of structural variations at the site of action and

the dynamic phenomena (absorption, distribution, metabolic destruction and excretion) that control drug concentration at that site.

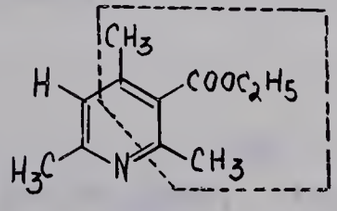
TABLE 4

BARBITURATE AND RELATED DRUGS THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

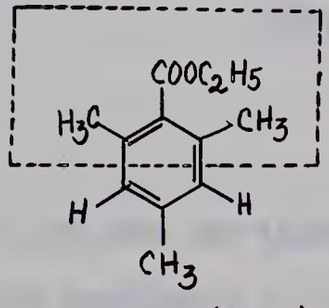
| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
| 5,5-diallyl-barbituric acid (XVIII) | 48.1 | 100 | +0.5 |
| | 9.6 | 20 | 0 |
| Seconal (XXXI) | 38.5 | 100 | +2.5 |
| | 1.9 | 5 | +0.5 |
| Isoniazid (XXXII) | 73.1 | 100 | 0 |
| α-allylmalonamide (XXXIII) | 70.4 | 100 | 0 |
| 2-propylpentanoyl-urea (XX) | 54.0 | 100 | +2 |
| | 27.0 | 50 | +1 |
| | 2.7 | 5 | trace |
| 2-allyl-2-isopropyl-acetamide (XVI) | 71.0 | 100 | +3 |
| | 14.2 | 20 | +2 |
| | 3.5 | 5 | +1 |
| | 0.7 | 1 | trace |
| 2-isopropylpent-4-enoylurea (XVII) | 54.3 | 100 | +2.5 |
| | 27.2 | 50 | +1 |
| | 2.7 | 5 | trace |

## PART Ic

A crucial question that remains to be explained is why two apparently chemically unrelated groups of drugs viz., those of the 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (X) series and those related to 2-allyl-2-isopropylacetamide (XVI) apparently act on the same site within the cell[21] and produce a very similar picture of experimental hepatic porphyria.[28] It was the purpose of the work reported in Part Ic of this thesis to explore this question. In Part Ia of this thesis it was concluded that the essential feature for porphyria-inducing activity in the 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine group of compounds was a sterically hindered ethoxycarbonyl substituent (as indicated by the groups within the areas demarcated by the dotted lines).

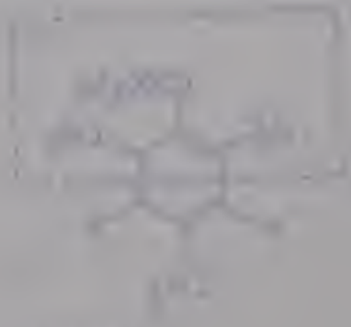



(XV)

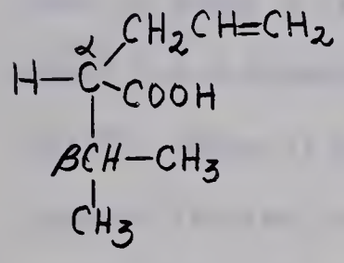



(XXIV)

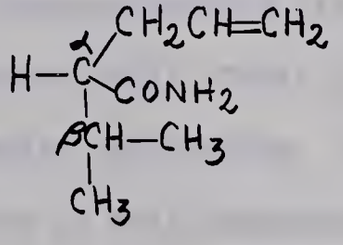
Although this group is not present in the 2-allyl-2-isopropylacetamide group of compounds, it is nevertheless of interest that in this group of compounds we have an amide group which is sterically hindered. Newman[29] has pointed out that the general impression gained on reading



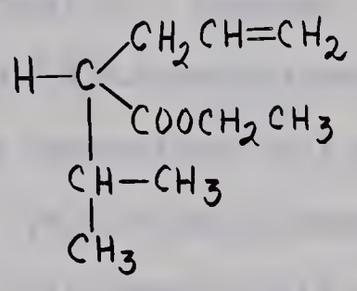
a variety of textbooks of organic chemistry is that branching at the $\alpha$-carbon atom is the most important factor in providing steric hindrance to esterification of aliphatic acids. He points out however, on the basis of considerable evidence, that it is branching at the $\beta$-carbon atom which is most decisive in providing steric hindrance to esterification. In 2-allyl-2-isopropylacetamide and related analogues there exists branching at the $\alpha$- and $\beta$-carbon atoms so that in the parent acid (2-allyl-2-isopropylacetic acid, XXXIV) considerable steric hindrance to esterification is to be expected.



(XXXIV)

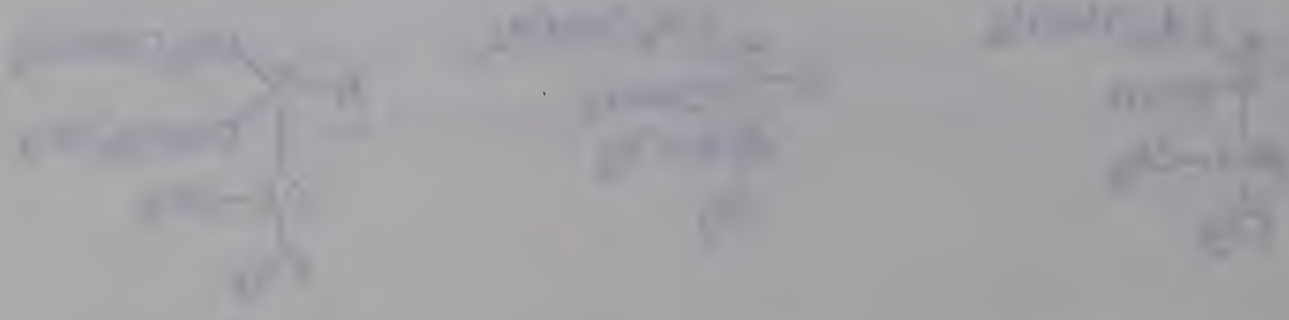


(XVI)



(XXXV)

As pointed out by Sykes,[30] the converse is also the case and therefore considerable hindrance will be anticipated in the hydrolysis of the ethyl ester of this acid (XXXV) and of the amide. It thus appeared possible that the underlying critical feature for porphyria-inducing activity in the 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine and 2-allyl-2-isopropylacetamide series of compounds was a sterically hindered ester or amide group. If this hypothesis is correct then it follows that the



amide and the ester groups should be interchangeable in these two groups of compounds with retention of activity. To test the correctness of this hypothesis the sterically hindered ester (ethyl 2,4,6-trimethylbenzoate, XXIV) and the sterically hindered amide (2-allyl-2-isopropylacetamide, XVI) were chosen as the critical compounds. The amide group of 2-allyl-2-isopropylacetamide was replaced by an ester group yielding the compound ethyl 2-allyl-2-isopropylacetate (XXXV) and the ester group of ethyl 2,4,6-trimethylbenzoate was replaced by an amide group giving 2,4,6-trimethylbenzamide (XXXVI). The porphyria-inducing activity of 2-allyl-2-isopropylacetamide (XVI, Table 5) was found to be similar to that of ethyl 2-allyl-2-isopropylacetate (XXXV, Table 5). Moreover, ethyl 2,4,6-trimethylbenzoate (XXIV, Table 5) and 2,4,6-trimethylbenzamide (XXXVI, Table 5) have similar activities. These observations this provide support for the correctness of this hypothesis. 2,4,6-Trimethylbenzamide was obtained from Marks and Bubbar and ethyl 2-allyl-2-isopropylacetate was synthesized by the following route: 2-allyl-2-isopropylacetamide (XVI) was treated with nitrous acid to give 2-allyl-2-isopropylacetic acid (XXXIV) which on treatment with ethanol and trifluoroacetic anhydride yielded the desired compound.

Many lipid soluble drugs are metabolized in the liver in such a manner that they become water soluble and can be excreted in the urine.[31] Granick[18] has recently suggested that the porphyrin biosynthetic pathway is involved in drug detoxication mechanisms. According to his view the drugs stimulate the formation of heme, and the heme formed may activate oxygen for hydroxylation reactions. This suggestion by Granick provides a possible

TABLE 5

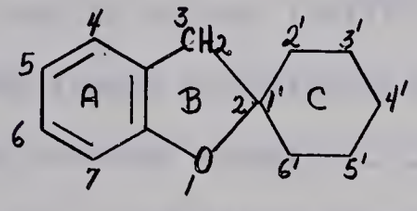
COMPOUNDS THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
| 2-allyl-2-isopropyl-acetamide (XVI) | 71.0 | 100 | +3 |
| | 14.2 | 20 | +2 |
| | 3.5 | 5 | +1 |
| | 0.7 | 1 | trace |
| ethyl 2-allyl-2-isopropyl-acetate (XXXV) | 62.4 | 100 | +2.5 |
| | 12.5 | 20 | +1 |
| | 3.1 | 5 | trace |
| | 0.6 | 1 | 0 |
| 2-allyl-2-isopropyl-acetic acid (XXXIV) | 71.0 | 100 | 0 |
| | 14.1 | 20 | 0 |
| ethyl 2,4,6-trimethyl-benzoate (XXIV) | 52.0 | 100 | +2.5 |
| | 5.2 | 10 | +1 |
| 2,4,6-trimethyl-benzamide (XXXVI) | 55.8 | 100 | +2 |
| | 5.6 | 10 | +1 |

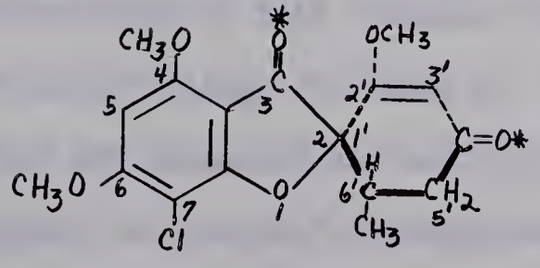
explanation for the requirement of a sterically hindered ester or amide group in the compounds described above. It is possible that because of the steric factors the active drugs cannot be solubilized by enzymic hydrolysis and that the porphyrin biosynthetic pathway is activated to participate in alternative mechanisms for solubilizing the drugs.

## PART Id

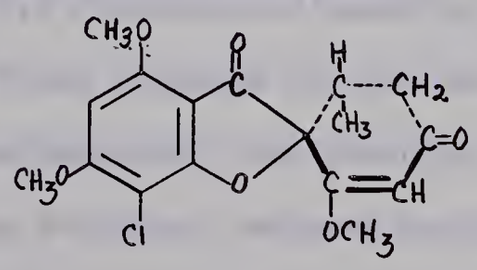
The structure of Griseofulvin (XXI) and the numbering system used are given below. The systematic nomenclature is based on the trivial name grisan for the tricyclic system (XXXVII) numbered as shown. Griseofulvin has two assymmetric carbon atoms which are located at positions 2 and 6'. The configuration at the two assymmetric centers 2 and 6' is designated by d or l. Griseofulvin is prefixed by (d,d), the optical antipode by (l,l), and the diasterioisomer (XL) epimeric at position 2 by (l,d); the spiran center (position 2) being that first mentioned.



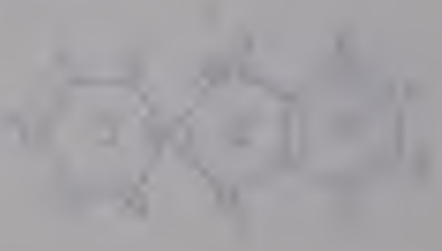

(XXXVII)

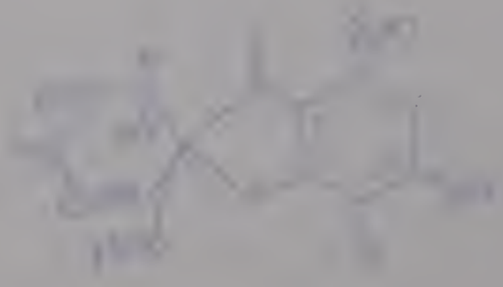


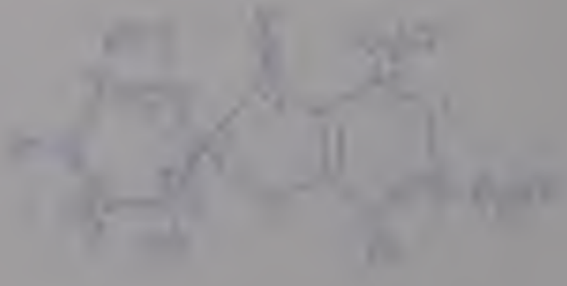

(d,d) Griseofulvin (XXI)




(l,d) Griseofulvin (XL)







As mentioned in the introduction, Granick has suggested that the critical feature in the Griseofulvin molecule that is required for porphyria-inducing activity is the distance between the keto oxygen atoms (starred in XXI). To investigate this hypothesis, and to determine the functional groups required in Griseofulvin for porphyria-inducing activity, Griseofulvin and various analogues have been tested in the chick embryo liver cell system and the results are recorded in Table 6. Griseofulvin and the analogues whose activities are recorded here were obtained through the courtesy of Dr. T.P.C. Mulholland of Imperial Chemical Industries, Great Britain.

The removal of the chlorine atom from position 7 of ring A of Griseofulvin does not affect its porphyria-inducing activity (see 4,6, 2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione, (XXXVIII, Table 6). Moreover, removal of the methoxy groups on positions 4 and 6 of ring A results in a markedly increased activity (7-chloro-2'-methoxy-6'-methylgris-2'-en'3,4'-dione, XXXIX, Table 6). This latter observation must be treated with caution since the stereochemistry of this molecule is different from that of Griseofulvin. Alteration of the stereochemistry of Griseofulvin from the (d,d) form to the (l,d) form does not result in diminution in the activity of this compound (7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione, XL, Table 6). In these three compounds the two keto atoms are preserved and activity remains undiminished, thus providing support for Granick's interpretation of the structural features required for activity.

In 7-chloro-4,6,4'-trimethoxy-6'-methylgris-3'-en-3,2'-dione, (XLI),

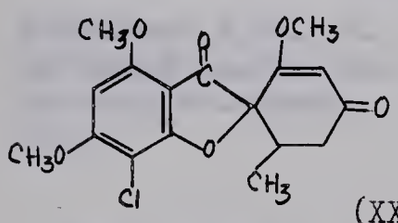
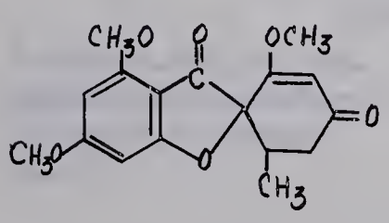
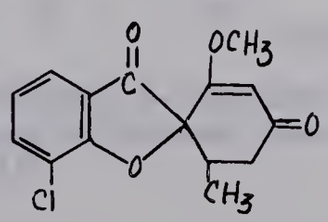
the positions of the keto and methoxy groups in ring C have been interchanged, thus bringing the keto groups closer together and resulting in reduced porphyria-inducing activity (Table 6). Removal of the keto group in ring C along with hydrogenation of the double bond and removal of the methoxy group at the 2' position, to give 7-chloro-4,6-dimethoxy-6'-methylgrisan-3-one (XLII), also results in a reduction in activity (Table 6). The results obtained with these two compounds serve to underline the importance of the position of the two keto groups and the distance between them.

The reduced activity of 7-chloro-4,6-dimethoxy-6'-methylgrisan-3,4'-dione (XLIII, Table 6) is apparently not in accord with Granick's hypothesis since the distance between the keto atoms appears to be maintained in the two-dimensional formula. However, construction of Dreiding Stereomodels showed that removal of the double bond provides ring C with increased flexibility so that the distance between the keto atoms probably differs from that in Griseofulvin. 7-Chloro-4,6-dimethoxy-6'-methylgris-2'-en-3,4'-dione (XLIV) was found to cause inhibition of growth and death of the cells at the higher dose level, as indicated by retraction of the smooth edge of the cell colonies and by a greatly decreased number of cells attached to the cover slip.[21] The activity exhibited by this compound at the lower dose level (Table 6) was not significantly different from that of Griseofulvin, indicating that the 2' methoxy group is not essential for porphyria-inducing activity.

It is of considerable interest that despite the differences in the porphyria-inducing activity of the Griseofulvin analogues, all of the

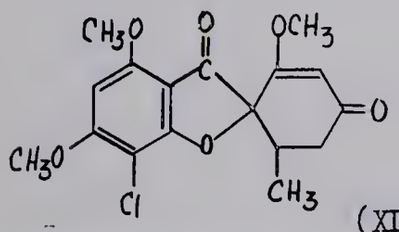
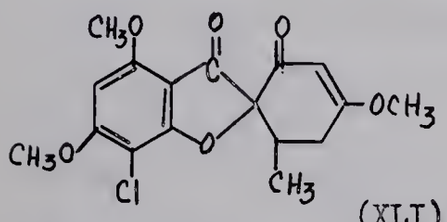
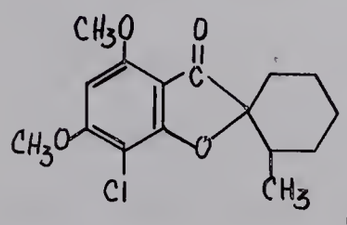
TABLE 6

GRISEOFULVIN AND VARIOUS ANALOGUES THAT INDUCE PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Concentration M x $10^{-5}$ | Concentration Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
|  (XXI) 7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) | 2.83<br>.57 | 10<br>2 | +2.5<br>+1 |
|  (XXXVIII) 4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) | 3.14<br>.63 | 10<br>2 | +2.5<br>+0.5 |
|  (XXXIX) 7-chloro-2'-methoxy-6'-methylgris-2'-en-3,4'-dione (dd,ll) | 3.42<br>.68 | 10<br>2 | +3.5<br>+2.5 |

(Table 6 cont'd)

TABLE 6 (cont'd)

| Compound Tested | Concentration M x $10^{-5}$ | Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
|  (XL) 7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en'3,4'-dione (l,d) | 2.83<br>.57 | 10<br>2 | +3<br>+1.5 |
|  (XLI) 7-chloro-4,6,4'-trimethoxy-6'-methylgris-3'-en-3,2'-dione (d,d) | 2.83<br>.57 | 10<br>2 | +1.5<br>+0.5 |
|  (XLII) 7-chloro-4,6-dimethoxy-6'-methylgrisan-3-one (d,d) | 3.23<br>.65 | 10<br>2 | +1<br>+0.5 |

(Table 6 cont'd)

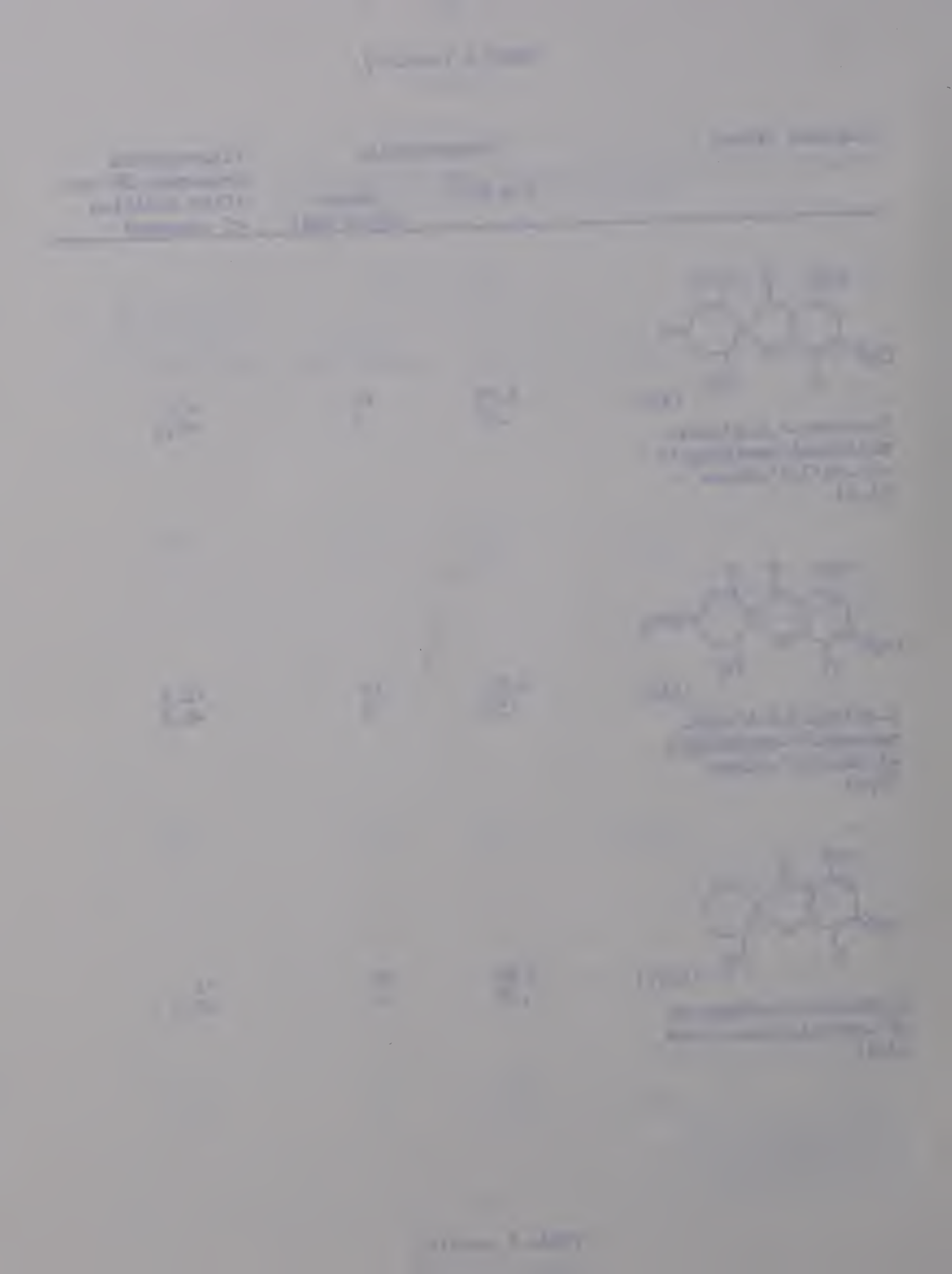
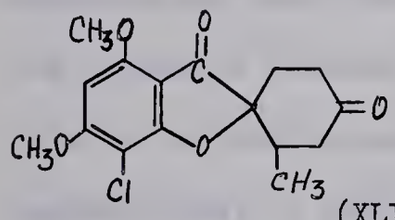
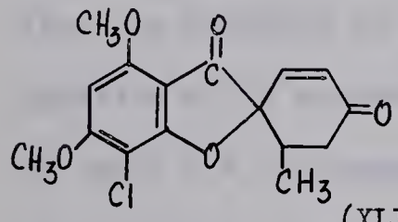
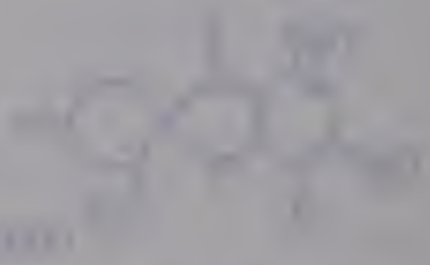


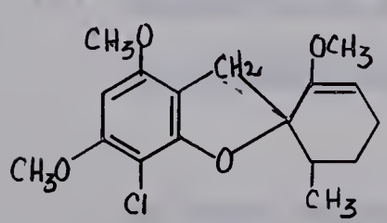
TABLE 6 (cont'd)

| Compound Tested | Concentration M x $10^{-5}$ | Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|---|
|  (XLIII) 7-chloro-4,6-dimethoxy-6'-methylgrisan-3,4'-dione (d,d) | 3.09<br>.62 | 10<br>2 | +1.5<br>trace |
|  (XLIV) 7-chloro-4,6-dimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) | 3.10<br>.62 | 10<br>2 | 0<br>+0.5 |

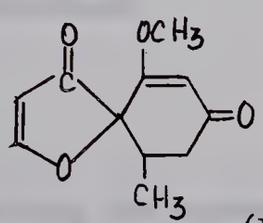


analogues display some activity. This finding contrasts with that observed in the pyridine series where small structural changes resulted in inactivity. It thus appears that the porphyria-inducing activity is inherent in the grisan-3-one structure. As a working hypothesis, we wish to suggest that the molecular feature responsible for activity in the Griseofulvin molecule is the keto group in the 3 position which is maintained in a plane perpendicular to that of ring C because of the stereochemistry of the spiran center 2. This molecular feature is analogous to the keto group in ethyl 2,4,6-trimethylbenzoate (XXIV) which is forced out of the plane of the ring by the two ortho methyl groups. We would further suggest that the activity in Griseofulvin is reinforced by the keto group in position 4' in an analogous fashion to the reinforcement of the activity of ethyl 2,4,6-trimethylbenzoate (XXIV) by the introduction of a second keto center in the molecule as in diethyl 2,3,5,6-tetramethylterephthalate (XXV).

The study of the porphyria-inducing activity of the following two compounds should prove decisive in checking the correctness of this hypothesis: (1) Compound (XLV) in which both keto groups have been reduced to methylene groups should be completely inactive. (2) Compound (XLVI), which lacks ring A of Griseofulvin but possesses both keto groups should retain the full activity of the parent molecule.




(XLV)




(XLVI)

# PART II

## (a) CONFIRMATION OF TAIT'S RESULTS

As mentioned in the introduction Tait[26] has reported that commercial avidin, a protein obtained from egg white, contains an impurity which inhibits δ-aminolevulinic acid synthetase (ALA synthetase) of Rhodopseudomonas spheroides. Because of its possible usefulness in porphyria, further studies of this inhibitor have been carried out and are reported in Part II of this thesis.

Our first experiments were directed to confirming the report of Tait on the presence of this inhibitor in commercial avidin. For this reason commercial avidin was added to a system containing glycine, succinyl-Coenzyme A and an ALA synthetase preparation from R. spheroides, and the amount of δ-aminolevulinic acid (ALA) formed was measured. Our results on the inhibitory power of commercial avidin (obtained from Nutritional Biochemicals Corporation) are summarized in Figure 4A and are similar to those reported by Tait (summarized in Figure 4B). Pure avidin, obtained from Dr. N.M. Green, National Institute for Medical Research, Mill Hill, England, had a very slight inhibitory power (Figure 4A) which is in agreement with the findings of Tait (Figure 4B).

## (b) DETERMINATION OF THE APPROXIMATE SIZE OF THE INHIBITOR OF δ-AMINOLEVULINIC ACID SYNTHETASE

Our next experiments were directed towards determining the approximate

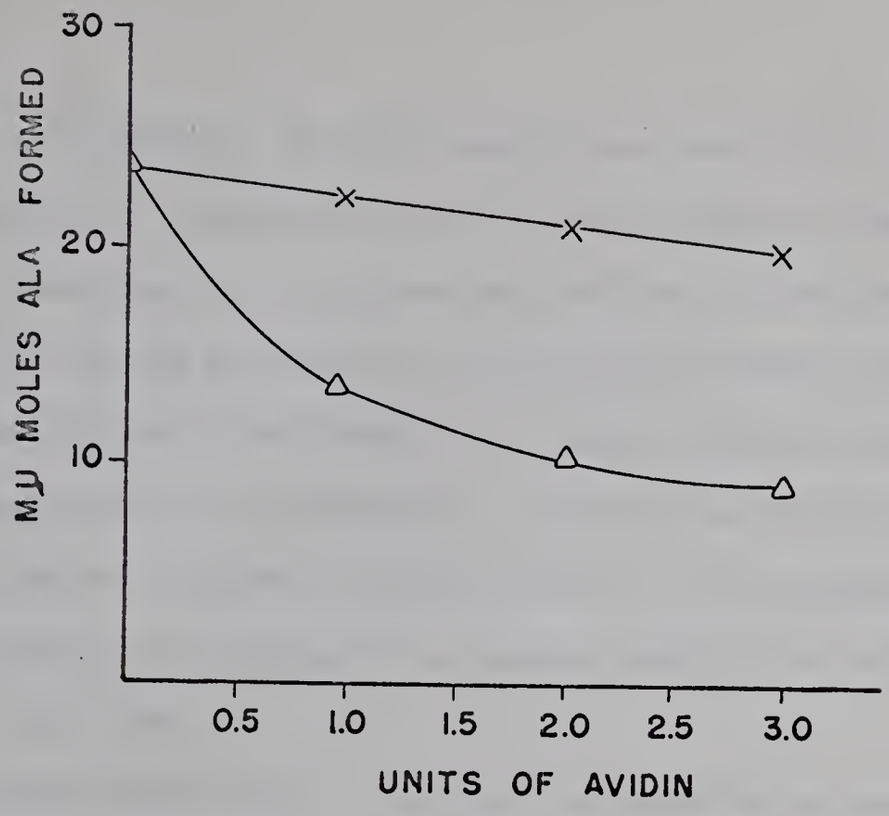



FIGURE 4A. Effect of avidin on ALA synthetase activity. The assay for ALA synthetase is described in the experimental section. Different quantities of avidin were preincubated with the enzyme extract for 10 min. at 0°. The reaction was started by addition of succinyl-CoA. △, Commercial avidin (2.5 units/mg. of protein); x, avidin (9.6 units/mg. of protein).

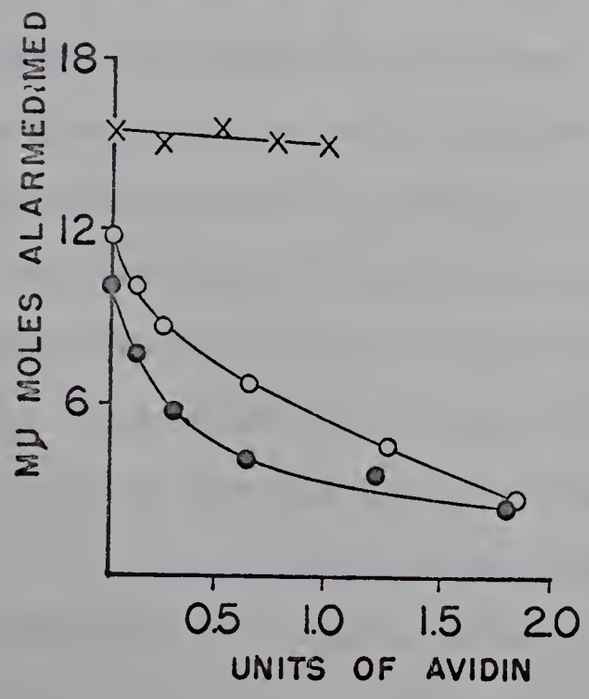



FIGURE 4B. Tait's Results, obtained under similar conditions ●, Commercial avidin (2.5 units/mg. of protein); ○, avidin (4.9 units/mg. of protein); x, avidin (9.6 units/mg. of protein).

size of the inhibitor. The first method used was that of dialysis. After a sample of commercial avidin had been dialyzed overnight, the material remaining in the dialysis bag and the dialysate were tested for their effect on ALA synthetase. The dialysate markedly inhibited ALA synthetase (Figure 5) indicating that a small molecular weight substance was responsible for this inhibition. The material which remained in the dialysis bag had a slight inhibitory effect on ALA synthetase, possibly because dialysis was incomplete or because some of the contaminant was bound to the avidin.

A second method used to determine the approximate size of the inhibitor found in commercial avidin was that of Sephadex fractionation. The dialysis experiments suggested that there were at least two components present in commercial avidin: one of higher molecular weight and therefore non-dialyzable, and one of low molecular weight which was dialyzable. Therefore it was thought that it would be valuable to confirm the finding obtained by dialysis using column chromatography with Sephadex gel as the packing substance for the columns. Sephadex consists of small grains of a hydrophilic insoluble substance made by cross linking the polysaccharide dextran. Sephadex acts like a molecular sieve, so that diffusion down through the column depends on the size of the molecules. Large molecules are prevented from entering the gel grains and pass down through the column first, while smaller molecules are able to move into the gel grains and thus move at a slower rate through the column.

A chromatographic column using Sephadex G-50 was set up and five milligrams of commercial avidin in phosphate buffer were added to the

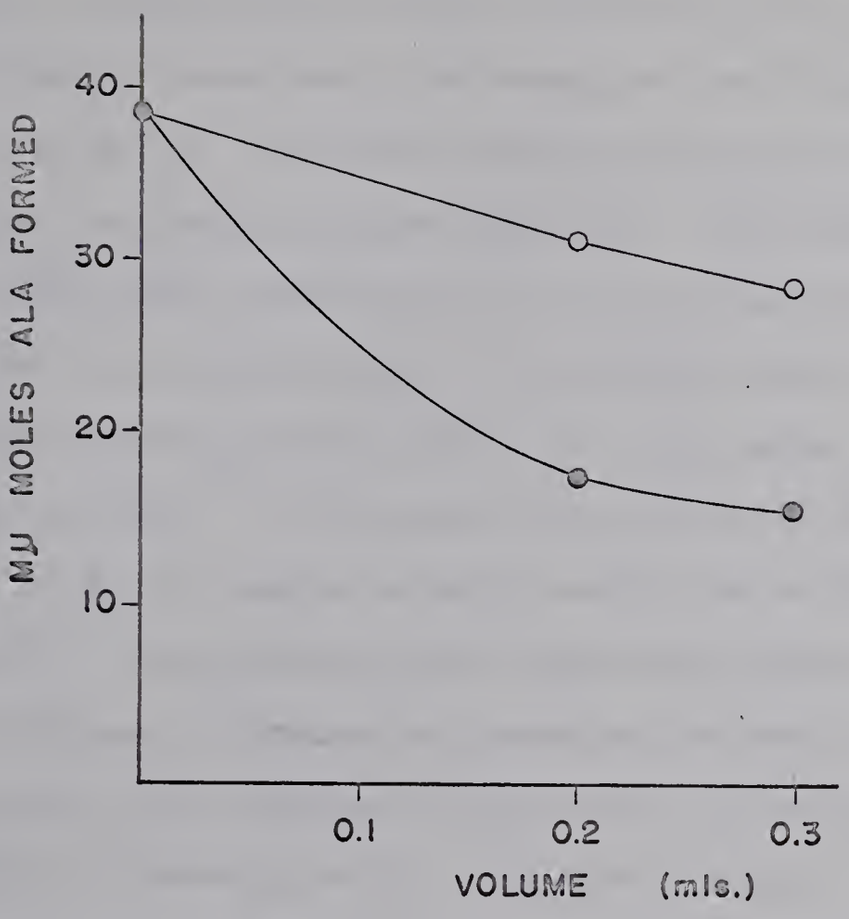



FIGURE 5. Inhibitory effect of Commercial Avidin on ALA synthetase after dialysis. The description of the dialysis of Commercial Avidin and the assay of ALA synthetase is given in the experimental section. ●, dialysate; ○, non-dialyzable material.

column. Eighteen fractions were collected and the optical density of each fraction was determined on the Bausch and Lomb Ultraviolet Spectrophotometer at 280 mμ. The optical densities of the fractions were plotted against the fraction numbers (Figure 6A). The component which comes off the column first (fractions 4 to 6) is the component of high molecular weight viz., purified avidin. The finding of high optical density in this component is the result of the high content of aromatic amino acids in avidin.[32] The component which comes off the column last (fractions 8 to 10) is of a smaller molecular weight than avidin (molecular weight 60,000).[32] The inhibitory power towards ALA synthetase of each of fractions one to eighteen was tested and the results are recorded in Figure 6B. This experiment revealed that two inhibitory components were present in commercial avidin. The first inhibitory component has an apparent molecular weight of the same order as avidin while the second component appears to have a considerably lower molecular weight.

To obtain a better estimate of the molecular weight of the inhibitory components, the elution pattern of these components from a column of Sephadex G-50 (Figure 7) was compared with the elution pattern obtained with a series of substances of known molecular weight viz., ovomucoid (molecular weight, 30,000), glycopeptide (molecular weight, 1500) and mannose (molecular weight, 180). Thus inspection of Figure 7 shows that the first inhibitory component of commercial avidin to emerge from the column has a molecular weight approximately that of ovomucoid. The second inhibitory component has a molecular weight of the same order of magnitude as the glycopeptide.

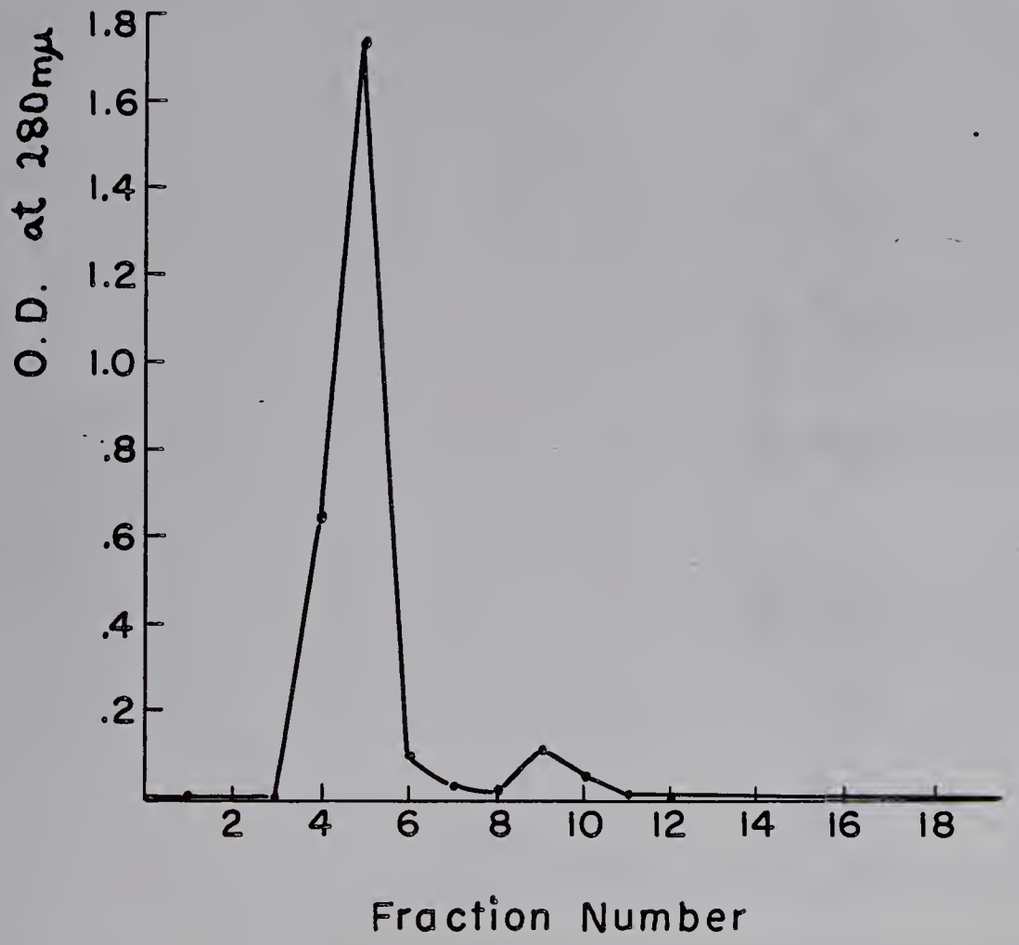



Figure 6A. Optical Density of Commercial Avidin fractions from a Sephadex G-50 column.

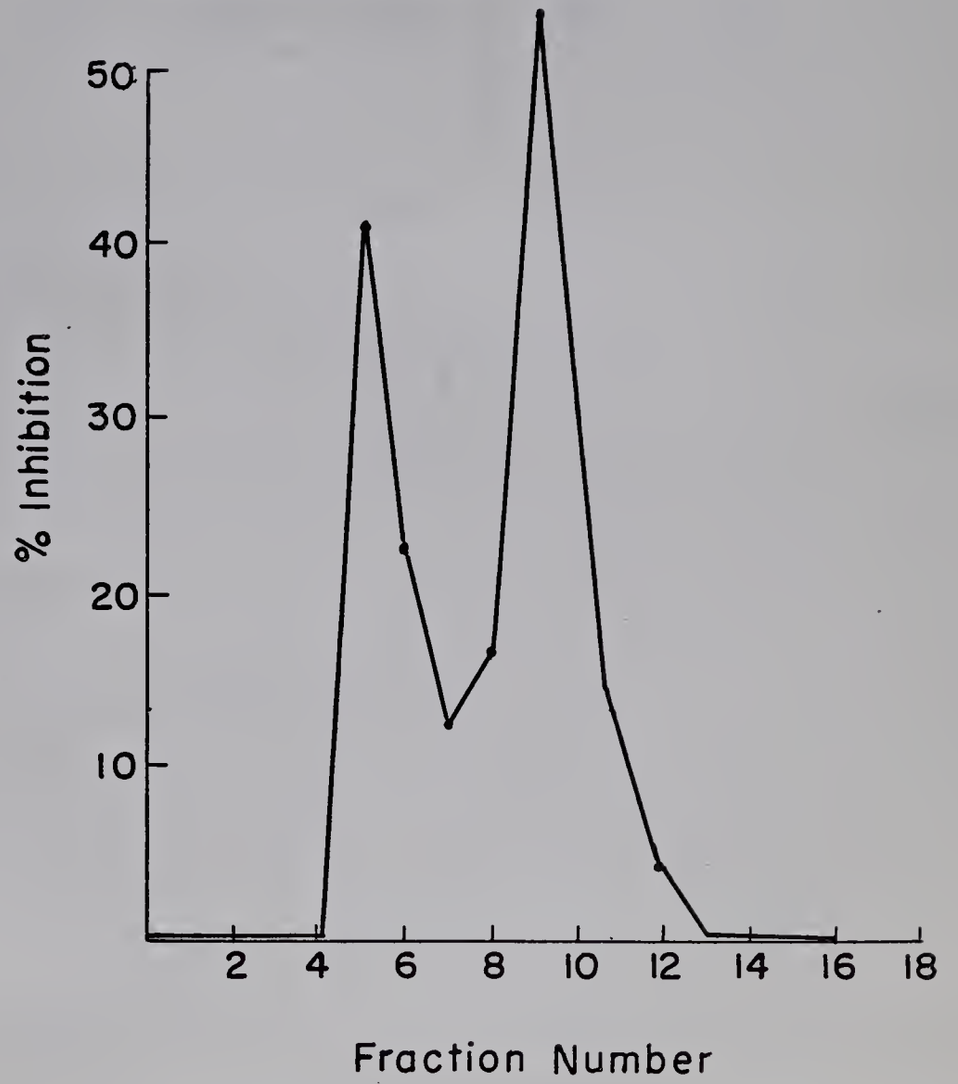



Figure 6B. Percentage Inhibition of ALA synthetase by Commercial Avidin fractions from a Sephadex G-50 column.

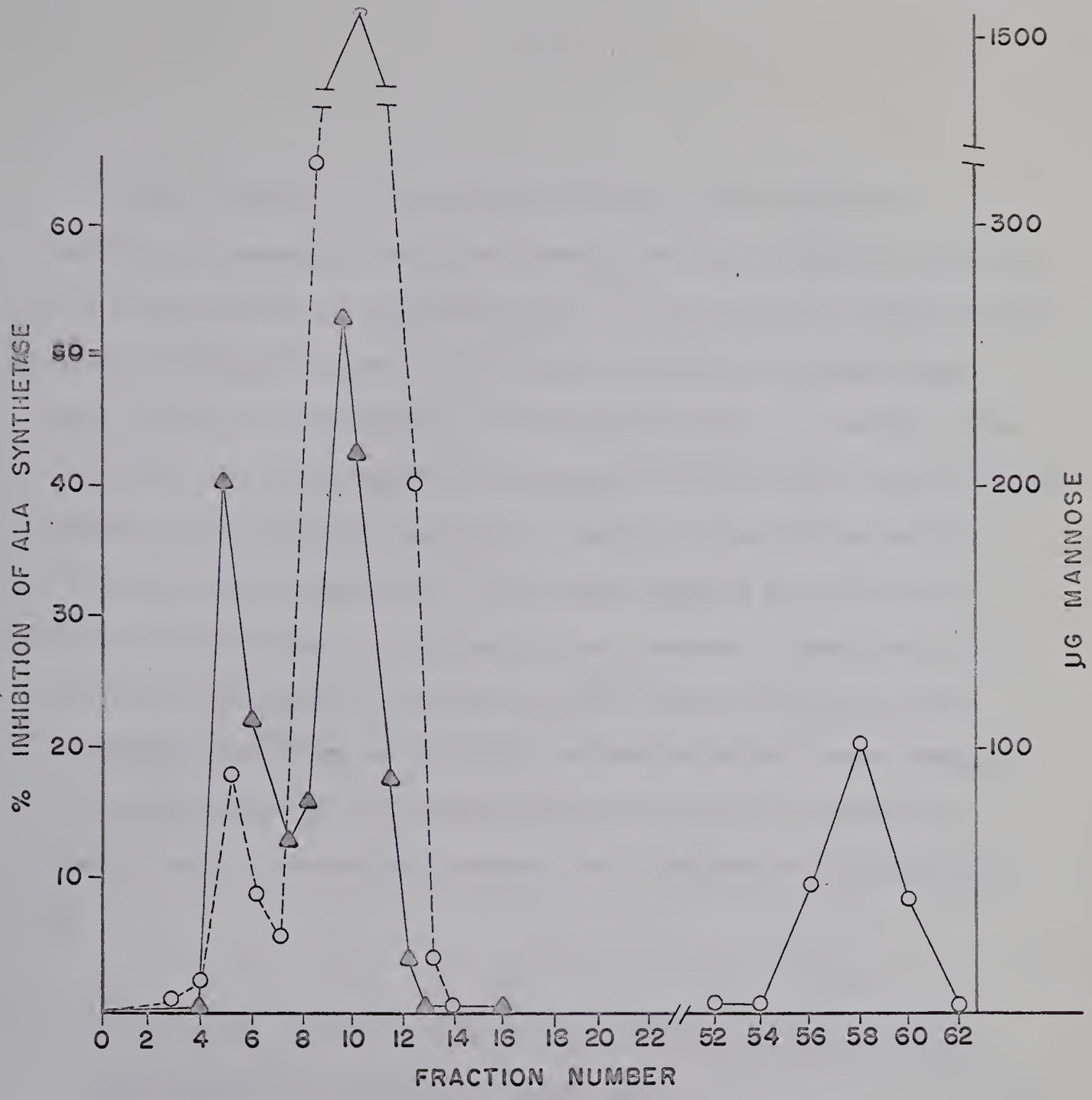



FIGURE 7. Gel-filtration on a column of Sephadex G-50. △, percentage inhibition of Commercial Avidin fractions; ○, the peaks from left to right are due to ovomucoid, glycopeptide and mannose respectively, as determined by orcinol-$H_2SO_4$ reaction (see experimental section for description).

A final estimate of the molecular weight of the inhibitory components of commercial avidin was carried out using Sephadex G-25 which has a lower porosity than Sephadex G-50. This was done for the following reason: Although Sephadex G-50 is able to efficiently separate molecules of high molecular weight, it has a high degree of porosity. Molecules of low molecular weight are therefore held up in the column and their molecular weight cannot be estimated accurately. The elution pattern of the inhibitory components of avidin were compared with the elution pattern obtained using the glycopeptide and mannose. Comparison of the positions of the peaks of Figures 8A and 8B indicate that the first component of inhibitory activity has a molecular weight larger than that of the glycopeptide as anticipated, while the second component has a molecular weight intermediate between that of mannose and the glycopeptide.

## (c) STUDY OF THE INHIBITORY ACTIVITY OF COMMERCIAL AVIDIN IN CHICKEN ERYTHROCYTES AND CHICK EMBRYO LIVER CELLS

The inhibitor present in commercial avidin has been tested on the ALA synthetase of *Rhodopseudomonas spheroides* and it was of interest to determine whether it might inhibit this enzyme in chicken erythrocytes and chick embryo liver cells.

### (i) Studies in Chicken Erythrocytes

Granick[33] has shown that chicken erythrocytes are able to synthesize protoporphyrin using glycine and $\alpha$-ketoglutarate as substrates

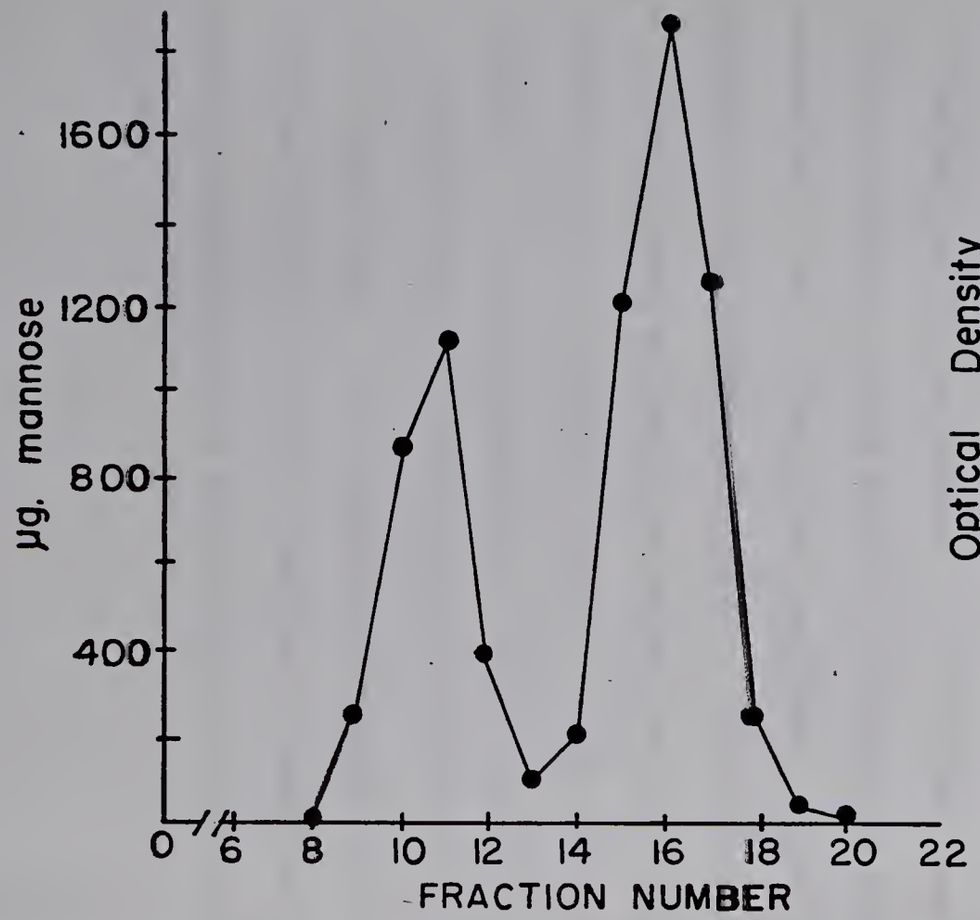



Figure 8A. Glycopeptide-mannose separation on a Sephadex G-25 column. ●, The peaks from left to right are due to glycopeptide and mannose (determined by orcinol-$H_2SO_4$ reaction, experimental section).

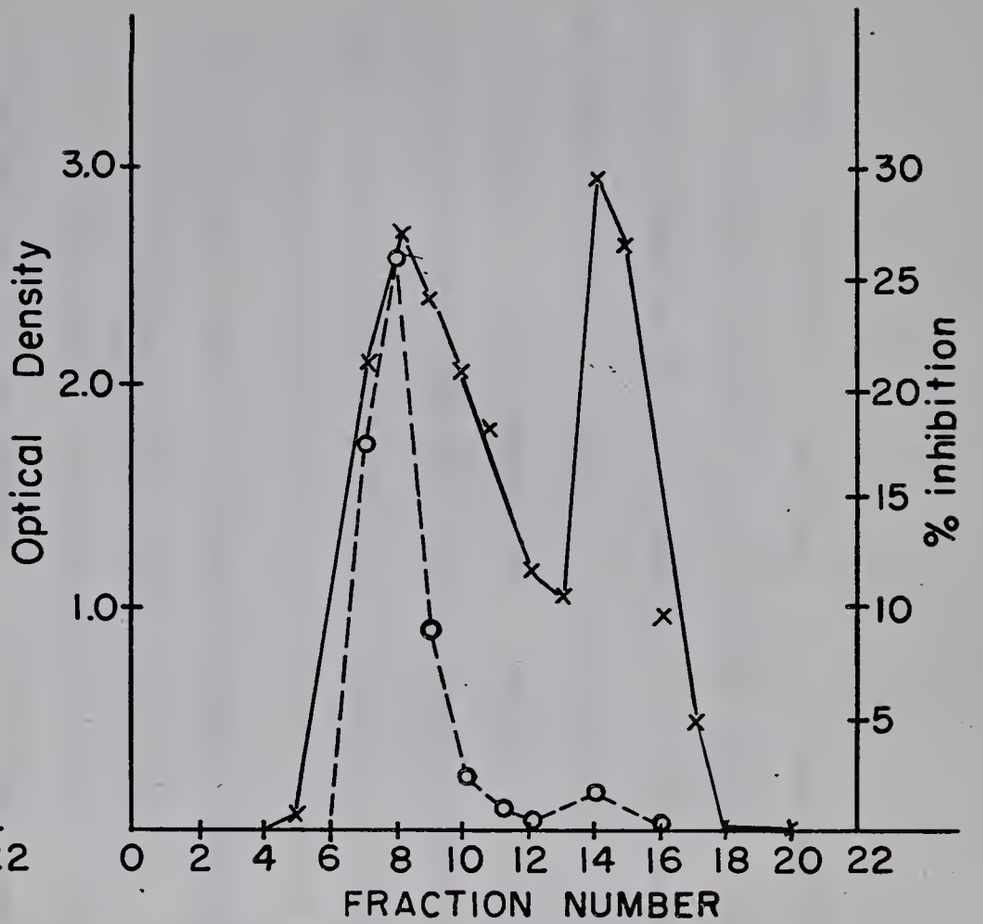



Figure 8B. o, Comparison of optical density and x, percentage ALA synthetase inhibition of avidin fractions using Sephadex G-25.

and that this synthesis proceeds in a linear manner for the first twenty hours. We have confirmed this finding (Figure 9) and have moreover shown that the formation of protoporphyrin from these substrates is not affected by commercial avidin (Figure 9), thus demonstrating that ALA synthetase was not inhibited in this experiment. The lack of inhibitory activity could be due to the fact that the inhibitory components present in commercial avidin were not able to penetrate the red blood cells. An alternate explanation is that ALA synthetase of avian red cells has different properties than the enzyme present in Rhodopseudomonas spheroides.

(ii) Studies in Chick Embryo Liver Cells

The possible inhibitory activity of commercial avidin on the ALA synthetase of chick embryo liver cells was also studied. Commercial avidin was added to chick embryo liver cells to which had been also added sub-maximal doses of the active porphyria-inducing compound 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (X). The addition of commercial avidin was found to have no inhibitory effect on the stimulation of porphyrin formation by this coupound (Table 7). When added alone or with 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine, commercial avidin was found to have no inhibitory effect on the growth of the liver cells or their attachment to the cover slips. The experiments reported in this section thus indicate that the inhibitory component present in commercial avidin probably does not possess any potential value in the treatment of human porphyria.

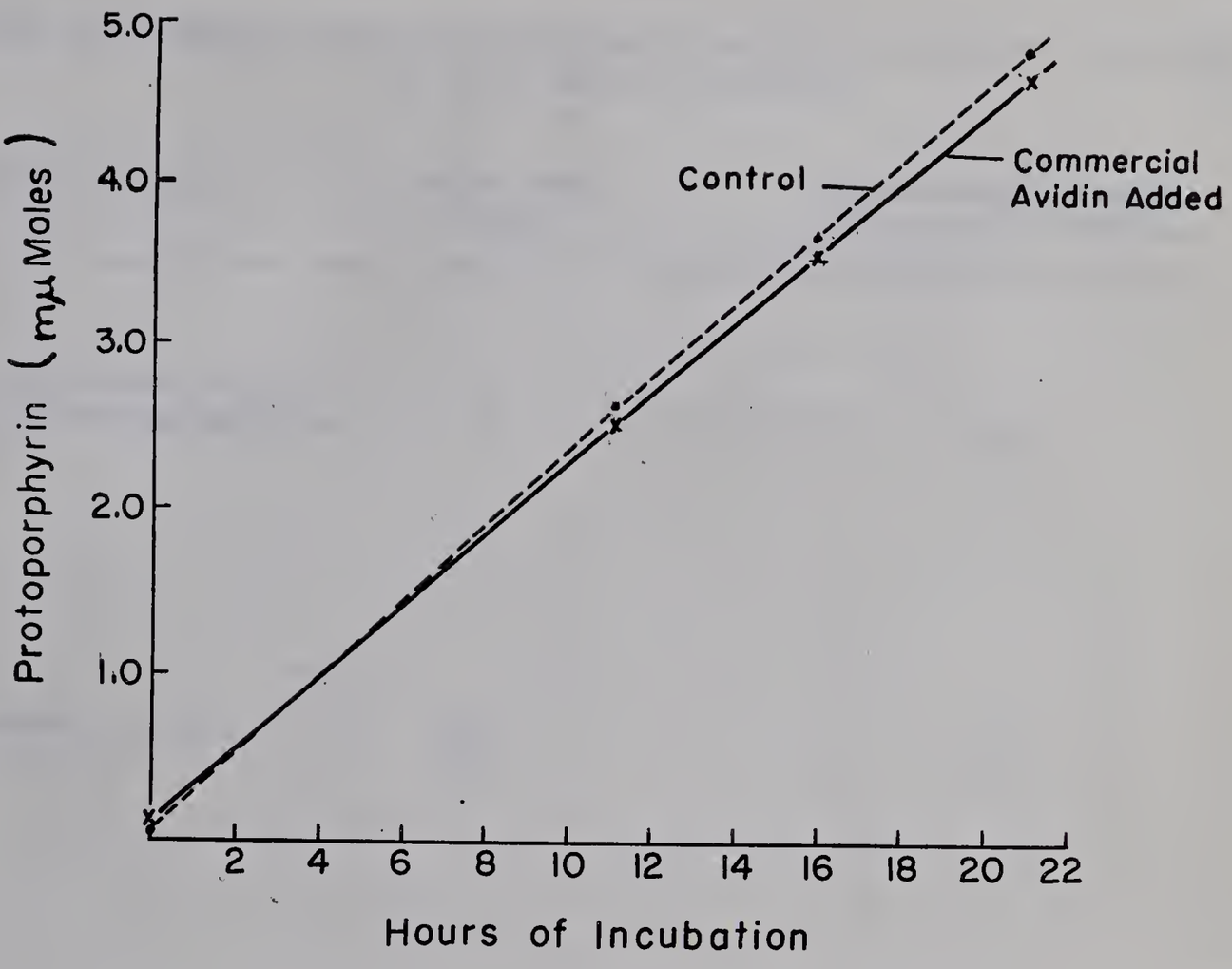



Figure 9. Effect of commercial avidin on the formation of protoporphyrin by chicken erythrocytes. The substrates used were 0.07 M glycine and 0.01 M α-ketoglutarate. Details on the preparation and incubation of the erythrocyte suspension and the extraction and estimation of protoporphyrin are given in the experimental section.

TABLE 7

EFFECT OF COMMERCIAL AVIDIN ON INDUCTION OF PORPHYRIN FORMATION IN CULTURES OF CHICK EMBRYO LIVER CELLS

| Compound Tested | Amount Added (µg) | Fluorescence Intensity 24 hrs. after addition of compound |
|---|---|---|
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine | 6 | +2.5 |
| commercial avidin | 500 | 0 |
| | 100 | 0 |
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine | 6 | +2.5 |
| plus | | |
| commercial avidin | 500 | |
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine | 6 | +2.5 |
| plus | | |
| commercial avidin | 100 | |

# EXPERIMENTAL SECTION

All C, H and N Analyses were Carried

Out by Dr. C. Daessle, Montreal

3,5-DIETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE (X)

The procedure used by Braude et al.[34] for the preparation of 3,5-diethoxycarbonyl-2,6-dimethylpyridine was modified for the preparation of this compound. A solution of 3,5-diethoxycarbonyl-1,4,-dihydro-2,4,6-trimethylpyridine (15 g., 0.056 M) and chloranil (27.6 g., 0.112 M) in tetrahydrofuran (1 l.) was kept at room temperature overnight. The solvent was removed by evaporation under reduced pressure and the residue treated with 1 N hydrochloric acid (600 ml.). The tetra-chloroquinol which was insoluble was removed by filtration, and the acidic solution neutralized with 1 N sodium bicarbonate solution whereupon the pyridine separated as an oil. The oil (12.3 g.) was dissolved in ether, the ethereal solution washed with water and dried (sodium sulfate). Distillation afforded the product as a colorless oil (12.3 g., 82.6%) b.p. 108°/0.2 mm. Marks et al.[22] record b.p. 138°/0.4 mm.

PICRATE OF 3,5-DIETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE

The picrate was prepared by adding a saturated solution of picric acid (2 ml.) in absolute alcohol to 3,5-diethoxycarbonyl-2,4,6-trimethyl-pyridine (100 mg.). Two recrystallizations from absolute alcohol yielded yellow needle-like crystals, m.p. 158-161° (Found C, 49.20; H, 4.81; N, 11.31%; $C_{20}H_{22}O_{11}N_4$ requires C, 48.56; H, 4.48; N, 11.35%).

3-ETHOXYCARBONYL-5-CARBOXY-2,4,6-TRIMETHYLPYRIDINE (XXIII)

The synthesis employed was that of Michael.[35] A solution of alcoholic

potassium hydroxide (200 ml.,0.126 M) was refluxed with 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (6.578 g.,0.126 M) for eight hours. The alcohol was removed by evaporation under reduced pressure and the residue was dissolved in water. The aqueous solution was shaken with ether to remove any unreacted di-ester. The brown-colored aqueous solution was brought to pH 3 with 1 N hydrochloric acid and evaporated to dryness. The residue was extracted with several portions of boiling alcohol. After concentrating the alcoholic solution the product separated as a white crystalline material. Recrystallization afforded the acid ester (3.24 g., 55.9%), m.p. 155-156°. Michael[35] records m.p. 157°. The acid ester was titrated with 0.02 N sodium hydroxide and required 5.23 ml. The calculated value for the pure acid-ester was 5.27 ml.

3-ETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE (XV)

This pyridine ester was prepared by decarboxylation of the acid-ester (XXIII, 2.0 g.). On heating the acid-ester slowly to 250° large quantities of carbon dioxide were given off, and at 250-260° a slightly yellow oil distilled over. Michael[35] records b.p. 255-256°. On redistillation a pale yellow oil (0.62 g., 38%), b.p. 83°/0.3 mm. was obtained.

PICRATE OF 3-ETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE

The picrate was prepared by adding a saturated solution of picric

acid (2 ml.) in absolute alcohol to 3-ethoxycarbonyl-2,4,6-trimethyl-pyridine (100 mg.). Two recrystallizations from absolute alcohol yielded fine yellow needle-like crystals, m.p. 141-144° (Found C,48.21; H, 4.09%; $C_{17}H_{18}O_9N_4$ requires C,48.32; H, 4.29%).

2-ALLYL-2-ISOPROPYLACETIC ACID (XXXIV)

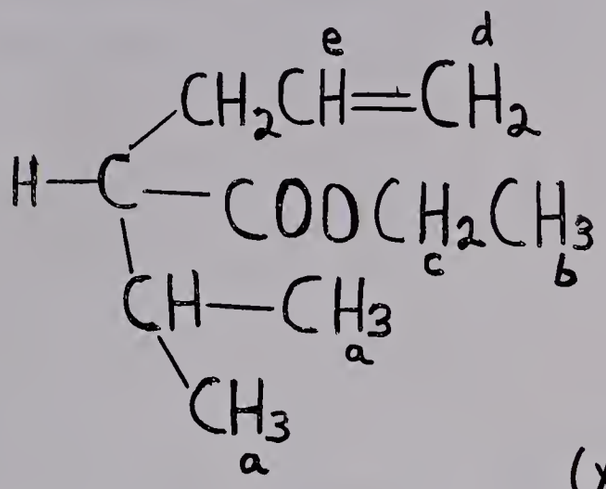
The method used by Whitmore and Langlois[36] for the preparation of acetic acid from acetamide was adapted for the preparation of this compound. 2-Allyl-2-isopropylacetamide (7.05 g., 0.05 moles) was suspended in water (200 ml.) at 55°. Sodium nitrite (3.45 g., 0.05 moles) and 40% sulfuric acid (32 ml.) were then added, and ten minutes later more sodium nitrite (3.45 g., 0.05 moles) and 40% sulfuric acid (32 ml.) was added. This mixture was then stirred for ten hours at 30-35°. The acidic product was extracted from the aqueous solution with three equal portions of ether, and then extracted from the ethereal solution with two equal portions of 7.5% sodium hydroxide. The sodium hydroxide layer was washed with ether four times to remove any unreacted amide and the pH of the sodium hydroxide layer was then brought to 4 by the addition of concentrated hydrochloric acid. The desired compound was extracted from the aqueous solution with three portions of ether. The ethereal solution was washed with water, dried (sodium sulfate), and distilled to remove the ether. Distillation afforded the desired product as a slightly yellow oil (1.3 g., 18.3%), b.p. 118°/0.3 mm. The acid thus obtained was titrated with 0.02 N sodium hydroxide and required 0.95 ml. The calculated value

for the pure acid was 0.94 ml.

ETHYL 2-ALLYL-2-ISOPROPYLACETATE (XXXV)

The method of Parish and Stock[37] was adapted for the preparation of this compound. Trifluoroacetic anhydride (7.5 ml., 0.054 moles) was added to a solution of absolute alcohol (0.52 ml., 0.009 moles) and 2-allyl-2-isopropylacetic acid (1.28 g., 0.009 moles); the resulting solution was allowed to stand at room temperature for two hours. Benzene (37.5 ml.) was added and the solution washed twice with 10% sodium hydroxide and with water. The benzene layer was dried (sodium sulfate) and the benzene removed by evaporation under reduced pressure. The residual oil was transferred to a bulb tube with ether. Distillation then afforded the product as a colorless oil (0.18 g., 12.2%), b.p. 95-100°/20 mm. Infrared (liquid film): Max. 1735 $cm^{-1}$ (ester carbonyl), 1645 $cm^{-1}$ (double bond), 1170 $cm^{-1}$ (isopropyl) (Figure 10A).

The nuclear magnetic resonance spectrum* is given in Figure 10B and observations regarding the spectrum are shown below.




(XXXV)

| Value | Proton Assignment | Relative Peak Area | |
|---|---|---|---|
| | | Found | Theoretical |
| 9.05 | a | 9.09 | 9 |
| 8.8 | b | | |
| 5.95 | c | 1.94 | 2 |
| 5.0 | d | 2.00 | 2 |
| 4.5 | e | 0.97 | 1 |

*Determined in carbon tetrachloride; external reference-tetramethylsilane; oscillator frequency 60 Mc.

INFRARED AND NUCLEAR MAGNETIC RESONANCE SPECTRA
OF ETHYL 2-ALLYL-2-ISOPROPYLACETATE

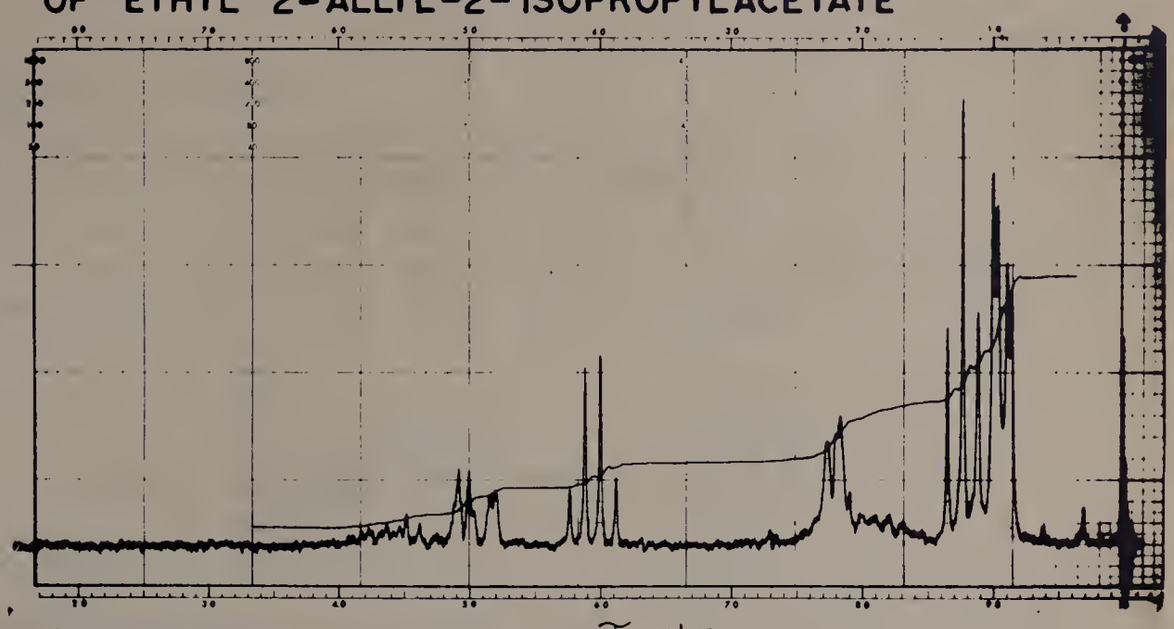



Figure 10 B

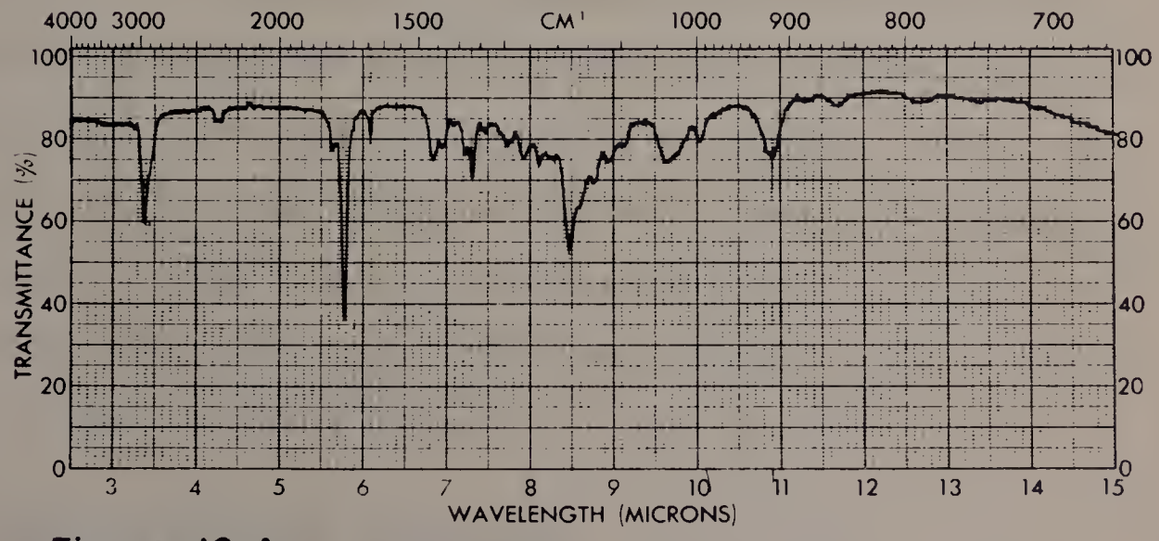



Figure 10 A

## TESTING OF COMPOUNDS FOR PORPHYRIA-INDUCING ACTIVITY IN CULTURES OF CHICK EMBRYO LIVER CELLS

(All procedures in this technique were carried out using standard microbiological aseptic technique)

### PREPARATION OF REAGENTS

#### (a) Calcium and Magnesium-free Earle's Medium

This medium contained the following substances dissolved in one litre of water and adjusted to pH 6.8 for storage:

| | |
|---|---|
| NaCl | 6.8 g. |
| KCl | 0.4 g. |
| $NaH_2PO_4.H_2O$ | 0.125 g. |
| Dextrose | 1.0 g. |
| $NaHCO_3$ | 2.2 g. |

This solution was sterilized by filtration through a Millipore filter (porosity 0.45u).

#### (b) Pangestin Solution

One gram of Pangestin (1:75 Difco certified), which is an active preparation of the enzymes of the pancreas (principally amylopsin, trypsin and steapsin) was added to calcium and magnesium-free Earle's medium (100 ml.) and allowed to stand for twelve hours. The pH was adjusted to 6.8 and the suspension filtered by gravity. The solution was finally filtered through a Millipore filter at 4°. The filtrate was divided into 5 ml. aliquots which were stored in screw cap culture tubes at -15°.

#### (c) Medium for Culturing Liver Cells

The following compounds were added to a 150 ml. Pyrex screw cap

bottle:

(i) 100 ml. Eagle's Basal Medium without phenol red (Microbiological Associates, cat. no. 12-104)

(ii) 1 ml. Glutamine solution (Microbiological Associates) (this solution was kept at -15° until used).

(iii) 10,000 U. buffered penicillin G. (crystalline potassium salt, Eli Lilly and Co.) dissolved in 0.25 ml. sterile water.

(iv) 10 mg. streptomycin sulfate (U.S.P. Eli Lilly and Co.) dissolved in 0.25 ml. sterile water.

(v) 2000 U. mycostatin (Squibb) dissolved in 0.25 ml. sterile water.

(vi) 10 ml. Fetal Bovine Serum (Microbiological Associates, cat. no. 14-413).

(d) Preparation of Enzyme Mixture for Digestion of Cells

Calcium and magnesium-free Earle's medium (6 ml.) was added to a vial containing sterile, crystallized and lyophillized trypsin (100 mg.). 3 Ml. of Pangestin solution were added to the vial and the contents of the vial mixed whereupon a clear solution resulted. This enzyme mixture was prepared following the removal of the liver from the chick embryo and was always used immediately after preparation.

PREPARATION OF LIVER CELLS

The liver from a seventeen day old chick embryo was washed three

times with calcium and magnesium-free Earle's medium in a small petri dish. The liver was transferred to a second petri dish containing the enzyme mixture and cut up into small pieces with a razor blade. After incubating the mixture on a serological bath at 37° until most of the cells were separated (15-30 min.), the suspension was transferred to a conical centrifuge tube whereupon the larger particles settled to the bottom of the tube. The suspension was then transferred to a second conical centrifuge tube. Aliquots (0.05 ml.) of the supernatant suspension of free cells were innoculated into aluminum capped culture vials (O.D. 19 x 63 mm.) containing a glass cover slip (size 5/8 inch, thinness 0) and 1 ml. of culture medium. The cells were incubated at 37° in an atmosphere of moist 95% air: 5% carbon dioxide for twenty-four hours.

COUNTING OF THE LIVER CELLS

Immediately after addition of the liver cells to the aluminum capped culture vials a small aliquot of the liver cell suspension was taken, diluted 1:100 in a serological pipette, and counted on a hematocytometer at a magnification of 25 diameters. The average number of cells found was $3\text{-}4 \times 10^5$ per 0.05 ml.

ADDITION OF CHEMICALS TO LIVER CELLS

The culture medium was removed from each culture vial by means of a Pasteur pipette and replaced with fresh medium (1 ml.). The chemicals under investigation were dissolved in 98% ethanol and added to the liver

cells by means of lambda pipettes. After the addition of the chemicals the culture vials were returned to the incubator for approximately twenty-four hours.

MEASUREMENT OF FLUORESCENCE INTENSITY

The cover slips were removed from the culture vials, inverted onto microscope slides and gently blotted dry with a filter paper. The cover slips were sealed to the slides with molten paraffin, washed with distilled water and examined for the presence of porphyrins with a fluorescence microscope.

RESULTS OBTAINED ON TESTING COMPOUNDS FOR PORPHYRIA-INDURING ACTIVITY

In all experiments carried out each compound was tested either in duplicate or triplicate, and the individual values obtained in a particular experiment are recorded in the following tables. Furthermore, most of the compounds were tested in at least two individual experiments. The value quoted for each compound in Tables 1 to 7 in the main section is the average of all the fluorescence values obtained for that compound. The compounds listed in Table 1A below correspond to compounds listed in Table 1 in the main section. Similarly, compounds listed in Tables 2A to 7A below correspond to those compounds listed in Tables 2 to 7 in the main section. The fluorescence intensity was scored as follows: +4, all colonies fluoresce intensely; +3, most colonies fluoresce intensely; +2, most colonies fluoresce partially; +1, some colonies fluoresce partially.

RESULTS OBTAINED ON TESTING COMPOUNDS FOR PORPHYRIA-INDUCING ACTIVITY IN THE TISSUE CULTURE SYSTEM

Table 1A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 | Expt. 3 |
|---|---|---|---|---|
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (X) | 10 | +3, +4, +3 | +3, +2½ | |
| | 6 | | +2½ | +3, +2½ |
| | 3 | +1½, +2, +1½ | 1½, 1½ | +1, 1½ |
| | 0.2 | | 0, 0 | 0, 0 |
| 3-ethoxycarbonyl-2,4,6-trimethylpyridine (XV) | 100 | | +3½, +3½ | |
| | 10 | +1½, +1, +1 | +1, +1, +1½ | |
| | 3 | trace(3) | trace | |
| | 1 | 0, 0, 0 | | |
| 2,4,6-trimethylpyridine (XIV) | 100 | | +1, +½ | +1, +1 |
| | 10 | | 0, 0 | 0, 0 |
| 3,5-diethoxycarbonylpyridine (XIII) | 100 | | 0, 0 | 0, 0 |
| | 10 | | 0, 0 | |
| 3,5-diethoxycarbonyl-4-methylpyridine (XII) | 100 | | 0, 0 | |
| | 10 | 0, 0, 0 | 0, 0 | |
| 3-ethoxycarbonyl-5-carboxy-2,4,6-trimethylpyridine (XXIII) | 100 | | 0, 0 | |
| | 10 | 0, 0 | 0, 0 | |

Table 2A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 |
|---|---|---|---|
| ethyl 2,4,6-trimethylbenzoate (XXIV) | 100 | +2½, +2½ | +3, +2½ |
| | 10 | +1, +½, +1 | +1, +1 |
| diethyl 2,3,5,6-tetramethyl-terephthalate (XXV) | 10 | +3½, +3, +3½ | +3, +4 |
| | 3 | | +2½, +2½, +2 |
| | 1 | +1, +1 | +1, +½ |
| dimethyl 2,3,5,6-tetramethyl-terephthalate (XXVI) | 10 | | +3½, +3½, +4 |
| | 1 | | +1½, +2, +1½ |
| dipropyl 2,3,5,6-tetramethyl-terephthalate (XXVII) | 10 | | +4, +3½, +4 |
| | | | +2, +2, +1½ |
| diisopropyl 2,3,5,6-tetra-methylterephthalate (XXVIII) | 10 | | +3½, +3, +3½ |
| | 1 | | +1½, +2, +1½ |
| tetramethylterephthalic acid (XXIX) | 100 | 0, 0 | |
| | 10 | 0, 0 | |
| 2,4,6-trimethylbenzoic acid (XXX) | 100 | | 0, 0 |
| | 10 | | 0, 0 |

Table 3A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 | Expt. 3 |
|---|---|---|---|---|
| 2-allyl-2-isopropyl-acetamide (XXVI) | 100 | +3,+3 | +2½,+3 | +3½,+3 |
| | 20 | +2,+2 | | +2,+1½ |
| | 5 | +½,+1 | +1,+1 | |
| | 1 | | trace,+½ | trace(2) |
| 2-propyl-2-isopropyl-acetamide (XIX) | 100 | +3½,+4 | +3½,+3½,+3 | +3½,+3 |
| | 20 | | +2,+2½ | |
| | 5 | +1,+1½ | +1,+1 | |
| | 1 | trace,+½ | | trace(2) |
| 2-isopropylpent-4-enoylurea (XVII) | 100 | +2½,+2 | +2½,+2½ | +2½,+2 |
| | 50 | +1,+1½ | +1,+1 | |
| | 5 | trace(2) | trace(2) | +½,+½ |
| 2-propylpentanoylurea (XX) | 100 | +2,+2 | +2,+1½ | +2,+1½ |
| | 50 | +1,+1½ | +1,+½ | +½,+1 |
| | 5 | +½,+½ | trace(2) | trace(2) |

Table 4A

| Compound Tested | Amount Added (µg) | Flourescence Intensity Expt. 1 | Expt. 2 | Expt. 3 |
|---|---|---|---|---|
| 5,5-diallylbarbituric acid (XVIII) | 100 | $+\frac{1}{2}, +1$ | $+\frac{1}{2}, +\frac{1}{2}$ | $+1, +\frac{1}{2}$ |
| | 20 | trace, 0 | 0, 0 | 0, trace |
| Seconal (XXXI) | 100 | $+3, +2\frac{1}{2}$ | $+2\frac{1}{2}, +2\frac{1}{2}$ | $+2, +2\frac{1}{2}$ |
| | 5 | $+1, +\frac{1}{2}$ | $+\frac{1}{2}, +\frac{1}{2}$ | trace, $+\frac{1}{2}$ |
| Isoniazid (XXXII) | 100 | 0, trace | | 0, 0 |
| α-allylmalonamide (XXXIII) | 100 | 0, 0 | | 0, 0 |
| 2-propylpentanoyl-urea (XX) | 100 | $+2, +2$ | $+2, +1\frac{1}{2}$ | $+2, +1\frac{1}{2}$ |
| | 50 | $+1, +1\frac{1}{2}$ | $+1, +\frac{1}{2}$ | $+\frac{1}{2}, +1$ |
| | 5 | $+\frac{1}{2}, +\frac{1}{2}$ | trace(2) | trace(2) |

Table 5A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 | Expt. 3 |
|---|---|---|---|---|
| 2-allyl-2-isopropyl-acetamide (XVI) | 100 | +3,+3 | +2½,+3 | +3½,+3 |
| | 20 | +2,+2 | | +2,+1½ |
| | 5 | +½,+1 | +1,+1 | |
| | 1 | | trace,+½ | trace(2) |
| ethyl 2-allyl-2-isopropyl-acetate XXXV) | 100 | +3,+2½ | +2½,+2½,+3 | +2½ |
| | 20 | | +1,+1 | +1½,+1 |
| | 5 | +½, 0 | trace(2) | |
| | 1 | | | 0, 0 |
| 2-allyl-2-isopropyl-acetic acid (XXXIV) | 100 | 0, 0 | 0, 0 | |
| | 20 | | 0, 0 | |
| ethyl 2,4,6-trimethyl-benzoate (XXIV) | 100 | +2½,+2½ | +3,+2½ | |
| | 10 | +1,+½,+1 | +1,+1 | |
| 2,4,6-trimethylbenz-amide (XXXVI) | 100 | | | +2,+2,+1½ |
| | | | | +½,+1,+1 |

Table 6A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 |
|---|---|---|---|
| 7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) (XXI) | 10 | +3, +2½, +2½ | +2½, +2, +2½ |
| | 2 | +1, +1 | +½, +1, +1 |
| 4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) (XXXVIII) | 10 | +2, +2½, +2½ | +2, +3, +2½ |
| | 2 | +½, +½ | +1, +½ |
| 7-chloro-2'-methoxy-6'-methylgris-2'-en-3,4'-dione (dd,ll) (XXXIX) | 10 | +4, +3½, +3½ | +4, +3½, +3½ |
| | 2 | +2½, +2 | +2, +2½, +2½ |
| 7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione (l,d) (XL) | 10 | | +3, +3, +3½ |
| | 2 | | +1½, +1½, +1 |
| 7-chloro-4,6,4'-trimethoxy-6'-methylgris-3'-en-3,2'-dione (XLI) | 10 | +1½, +1, +1½ | +2, +1½, +1½ |
| | 2 | +½, +½ | +1, +½, +½ |
| 7-chloro-4,6-dimethoxy-6'-methylgrisan-3-one (d,d) (XLII) | 10 | | +1, +1½, +1 |
| | 2 | | +½, +½, +½ |

(Table 6A cont'd)

Table 6A (cont'd)

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 |
|---|---|---|---|
| 7-chloro-4,6-dimethoxy-6'-methylgrisan-3,4'-dione (d,d) (XLIII) | 10 | | +1½, +1½, +1 |
| | 2 | | +½, trace(2) |
| 7-chloro-4,6-dimethoxy-6'-methylgris-2'-en-3,4'-dione (d,d) (XLIV) | 10 | | 0, 0, 0 |
| | 2 | | +1, +½, +½ |

Table 7A

| Compound Tested | Amount Added (µg) | Fluorescence Intensity Expt. 1 | Expt. 2 |
|---|---|---|---|
| commercial avidin | 500 | | 0, 0 |
| | 100 | | 0, 0 |
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (X) | 6 | | +2½, +2½ |
| plus | | | |
| commercial avidin | 500 | | |
| 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (X) | 6 | | +2½, +2½ |
| plus | | | |
| commercial avidin | 100 | | |

MAINTENANCE OF RHODOPSEUDOMONAS SPHEROIDES

R. spheroides (N.C.I.B. No. 8253) obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, was maintained at 4° in the dark in stab culture. The agar slants for these stab cultures were prepared as follows: Difco agar (1.5 g.) and Difco yeast (300 mg.), were added to 150 ml. of Medium S of Lascelles[38]; the mixture was heated to dissolve the contents and aliquots (5 ml.) of the warm solution added to test tubes (150 x 25 mm.). Using sterile precautions the R. spheroides cells were subcultured monthly by transferring them to fresh agar slants. The cells were then grown for 40 hours at 32 - 34° in the light. A tungsten lamp (60W) was adjusted so that the intensity of the light falling on the cultures was 250 foot-candles. Medium S of Lascelles[38] contained the following substances dissolved in one litre of water and adjusted to pH 6.8 for storage:

| | |
|---|---|
| L-glutamic acid | 2.98 g. |
| Malic acid | 2.70 g. |
| $KH_2PO_4$ | 0.50 g. |
| $K_2HPO_4$ | 0.50 g. |
| $(NH_4)_2PO_4$ | 0.80 g. |
| $MgSO_4.7H_2O$ | 0.20 g. |
| $CaCl_2$ | 0.04 g. |
| Nicotinic acid | 0.001 g. |
| Thiamine HCl | 0.001 g. |
| Biotin | 10 µg. |

Determination of Extracellular Enzyme Activity

Cellulase [E.C.3.2.1.4, EC 232] activity from the following Cellulolytic or Terrestrial Bacterial Isolates, Bacillus, was monitored at 37°C in the test tubes following. The assay mixture (1.0 g.) was prepared [50% wt.%] were added in [10 ml, or mixture 3 of Lamotte 36 Solution was heated to dissolve the contents and allowed (1 wt.) of the were solutions added to each tubes (1:2 x 2) Dil. Using sterile pipettes the 2% substrate until pure (additional control) or inoculate tubes to (test tube slants). The cells were then to be tubes at 37°C in the bath. A thermometer tube (pH) was adjusted as that the temperature of the tube. Following as the solutions was in Centrifuge, Section 1 of Lamotte Separation the following procedure to the Tube Analysis.

| | |
|---|---|
| Substrate Unit | 0.02 g. |
| Cells Wall | 0.01 g. |
| $KH_2PO_4$ | 0.0 g. |
| $K_2HPO_4$ | 0.0 g. |
| $(NH_4)_2SO_4$ | 2.0 g. |
| $MgSO_4.7H_2O$ | 0.20 g. |
| $MnCl_2$ | 0.00 g. |
| Distilled H2O | 2.001 g. |
| Solution #2 | 0.001 g. |
| Agar | 10 gm. |

GROWTH OF RHODOPSEUDOMONAS SPHEROIDES

Using a platinum loop, R. spheroides cells were transferred from an agar slant to several test tubes (100 x 16 mm.) containing yeast growth medium (4 ml.) of Lascelles[38] which was prepared by dissolving Difco yeast (80 mg.) in Medium S (40 ml.). The test tubes were placed in an oven and maintained at 32-34° for 24 hours using a tungsten lamp (60W) to provide illumination (250 foot-candles). 1.8 Ml. of this medium was transferred with a sterile pipette to an Erlenmeyer flask (500 ml.) containing Medium S (450 ml.) and the flask kept for 44 hours at 32-34° and 250 foot-candles light intensity. The cells grown in the above manner were then used for the preparation of ALA synthetase.

PREPARATION OF ALA SYNTHETASE OF RHODOPSEUDOMONAS SPHEROIDES[26]

The cells obtained as described above were centrifuged at 4° (1000 g, 10 minutes), washed with cold 0.05 M phosphate buffer, pH 7.4 (250 ml.) and then suspended in a minimum amount of 0.05 M phosphate buffer. This suspension was used at once or kept frozen at -15° until required. The suspension of organisms was disrupted in a Hughes Press, diluted with two volumes of 0.05 M phosphate buffer and centrifuged for 90 minutes at 105,000 g in the Spinco Model L Ultracentrifuge. The resulting clear supernatant containing the ALA synthetase was kept at -15° until required, and then used as such without further purification. The concentration of protein in the clear supernatant was measured by the Biuret reaction which is described below.

## BIURET REACTION [39]

### Biuret Reagent

Copper sulfate (1.5 g.) and sodium potassium tartrate (6.0 g.) were dissolved in water (500 ml.). Ten percent sodium hydroxide (300 ml.) was added slowly and the mixture stirred. The resulting solution was diluted to one litre with water.

### Procedure

A series of test tubes were set up containing 0 to 1 mg./ml. of crystalline bovine albumin (Armour Pharmaceutical Company) and Biuret reagent (0.4 ml.). Aliquots (0.2 ml.) of the clear supernatant containing ALA synthetase were added to two other test tubes, diluted to 1 ml. with water and treated with Biuret reagent (0.4 ml.). The test tubes were shaken and after 30 minutes the optical density at 550 mμ determined in a Unicam SP 600 spectrophotometer. A standard curve was constructed by plotting the optical densities of the crystalline bovine albumin standards against the concentration of these standards. From this curve the concentration of protein in the clear supernatant was determined.

## ASSAY OF δ-AMINOLEVULINIC ACID SYNTHETASE [26]

To an aliquot of the clear supernatant solution (equivalent to 0.05 to 0.3 mg. of protein) containing ALA synthetase was added phosphate buffer (pH 7.4, 37.5 μmoles), glycine (12.5 μmoles), succinyl-CoA (0.2 μmoles) and pyridoxal phosphate (0.05 μmoles). The final volume of all tubes was brought to 0.695 ml. with water and incubation was at 37°

for 30 minutes. The reaction was stopped by the addition of 0.145 ml. of 44% (w/v) trichloracetic acid. The precipitated protein was removed by centrifuging and washed with 0.7 ml. of 5% (w/v) trichloracetic acid. The combined supernatants were brought to pH 4.6 with 1 N sodium hydroxide and the ALA content determined as follows: To a test tube was added the deproteinized solution (1.0 ml.), acetate buffer, pH 4.6 (1.0 ml.) and acetylacetone (0.1 ml.). This solution was heated at 100° for 10 minutes, cooled, and Ehrlich-Hg reagent (2.0 ml.) added. After 15 minutes, the optical density at 552 mμ was determined with a Unicam SP 600 spectrophotometer. The amount of ALA formed per mg. of protein varied between 204.4 and 290.3 mμ moles per hour. The activity of ALA synthetase was expressed as the mμ moles of ALA formed per mg. of protein per hour.[26] The amount of ALA present was determined from the value of the Emax ($7.2 \times 10^4$).[40]

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SYNTHESIS OF SUCCINYL-COENZYME A

Coenzyme A (Sigma Chemical Company) was dissolved in ice cold water (3.0 ml.). To this solution was added succinic anhydride (0.3 mg.) followed by sodium bicarbonate until the pH was 7-7.5. The mixture was kept in an icebath and shaken frequently for 30 minutes, after which time a clear solution formed. The preparation was unstable and was therefore used immediately.

PREPARATION OF BUFFERS

Phosphate buffer of pH 7.4 was made by adding $NaH_2PO_4.H_2O$ (19 ml., 0.05 M) to $Na_2HPO_4.7H_2O$ (81 ml., 0.05 M) and diluting to 200 ml. Acetate buffer of pH 4.6 was made by adding glacial acetic acid (57 ml., 1 mole) to sodium acetate (136 g., 1 mole) and diluting to one litre.

PREPARATION OF EHRLICH - Hg REAGENT

This reagent contained the following substances: glacial acetic acid (168 ml.), 70% perchloric acid (40 ml.), dimethylaminobenzaldehyde (4.0 g.), mercuric chloride (0.7 g.) and water to make 220 ml.

AVIDIN

The pure avidin used contained 9.6 units per milligram of protein. Avidin is known to specifically bind biotin, so that one unit of avidin is defined as that amount of avidin binding one microgram of biotin. The commercial avidin used had an activity of 3.3 units per milligram of protein. When determining the effect of avidin on ALA synthetase

activity, it was preincubated with the enzyme for 10 minutes at 0°.

DIALYSIS OF COMMERCIAL AVIDIN

Commercial avidin (5 mg.) was dissolved in water (5.0 ml.) and dialyzed overnight against water (50 ml.). The contents of the dialysis bag and the dialysate were freeze-dried and each residue dissolved in water (1.32 ml.). Aliquots (0.2 ml. and 0.3 ml.) of the solutions were tested for their possible inhibitory action on ALA synthetase.

SEPHADEX G-25 AND G-50 COLUMNS

Sephadex G-25 or G-50 was stirred into a beaker containing 0.2 M $Na_2HPO_4 . 7H_2O$ (150 ml.) and allowed to stand for one hour. After sedimentation and decantation to remove fine gel particles the suspension was added slowly to a chromatographic column (0.8 x 26 cm.) which was partly filled with water. When a layer of 2-5 cm. of Sephadex had sedimented at the bottom of the column, a screw clip attached to the rubber tubing at the base of the column was adjusted to allow the water to flow slowly from the column. The remaining Sephadex slurry was added until the desired column height was reached (26 cm.). The column was washed with 0.2 M $Na_2HPO_4 . 7H_2O$ and the solution to be chromatographed (dissolved in 0.2 M $Na_2HPO_4 . 7H_2O$) was applied to the top of the Sephadex column. 0.2 M $Na_2HPO_4 . 7H_2O$ was used to elute the material from the column and the eluate collected in 2 ml. fractions using a RadiRac LKB Fraction Collector.

SEPARATION AND MEASUREMENT OF COMPONENTS OF COMMERCIAL AVIDIN

For separation of commercial avidin on either columns of Sephadex G-25 or Sephadex G-50, commercial avidin (5 mg.) was dissolved in 1.5 ml. of 0.2 M $Na_2HPO_4 . 7H_2O$, applied to the top of the Sephadex column and fractions collected as described above. The optical density of each fraction at 280 mu was determined on the Bausch and Lomb Ultraviolet Spectrophotometer. The pH of each fraction was brought to 7.4 by the addition of 0.4 M $NaH_2PO_4 . H_2O$ (0.32 ml.) since this was the optimal pH for ALA synthetase activity. An aliquot (1 ml.) of each fraction was added to the system described for the assay of ALA synthetase (0.695 ml.). The mixture was incubated at 37° for 30 minutes and the amount of ALA formed was measured as previously described. As a control an aliquot of the $Na_2HPO_4 . 7H_2O$ solution, collected from the column prior to the addition of avidin, was adjusted to pH 7.4 and 1 ml. added to the system described for the assay of ALA synthetase. The difference between the control value and the values obtained with the individual fractions was a measure of the inhibition exerted by each individual fraction. The percentage inhibition caused by individual fractions was calculated by dividing this difference by the value obtained in the control. The results are shown in Table 8 and Figure 6B.

TABLE 8

INHIBITION OF δ-AMINOLEVULINIC ACID SYNTHETASE BY COMMERCIAL AVIDIN FRACTIONS FROM A SEPHADEX G-50 COLUMN

| Fraction Number | Optical Density | Optical Density of Control Minus Optical Density of Fraction | % Inhibition |
|---|---|---|---|
| control | 0.245 | 0 | 0 |
| control | 0.245 | 0 | 0 |
| 3 | 0.245 | 0 | 0 |
| 4 | 0.245 | 0 | 0 |
| 5 | 0.145 | 0.100 | 41.0 |
| 6 | 0.190 | 0.055 | 22.5 |
| 7 | 0.215 | 0.030 | 12.3 |
| 8 | 0.205 | 0.040 | 16.3 |
| 9 | 0.115 | 0.130 | 53.1 |
| 10 | 0.195 | 0.050 | 20.4 |
| 11 | 0.215 | 0.030 | 12.2 |
| 12 | 0.235 | 0.010 | 4.1 |
| 13 | 0.245 | 0 | 0 |
| 14 | 0.245 | 0 | 0 |
| 15 | 0.245 | 0 | 0 |
| 16 | 0.245 | 0 | 0 |
| 17 | 0.245 | 0 | 0 |
| 18 | 0.245 | 0 | 0 |

## SEPARATION OF OVOMUCOID, GLYCOPEPTIDE AND MANNOSE ON SEPHADEX COLUMNS

### (i) Sephadex G-50

A solution of ovomucoid (5 mg.), glycopeptide (5 mg.) and mannose (1.5 mg.) in 0.2 M $Na_2HPO_4 . 7H_2O$ was applied to the top of a Sephadex G-50 column. The glycopeptide used was obtained by proteolytic digestion of the glycoprotein egg albumin by Marks et al.[42] Fractions of 2 ml. volume were collected from the Sephadex G-50 column. An orcinol-sulfuric acid reaction[43] was carried out on each fraction to determine the position of the ovomucoid, glycopeptide and mannose peaks. Mannose is a constituent common to these three substances and by means of the orcinol-sulfuric acid reaction the mannose content of these substances can be determined.

### (ii) Sephadex G-25

A solution of glycopeptide (5 mg.) and mannose (1.5 mg.) in 0.2 M $Na_2HPO_4 . 7H_2O$ was applied to the top of a Sephadex G-25 column and thirty fractions of 1.0 ml. volume were collected. An orcinol-sulfuric acid reaction was carried out on each fraction to determine the position of the glycopeptide and mannose peaks.

## ORCINOL-SULFURIC ACID REACTION FOR THE DETERMINATION OF MANNOSE

The method described by Winzler[43] was used to determine the mannose content of the fractions obtained from the Sephadex columns described above. The following reagents were required:

(i) Reagent A: concentrated sulfuric acid (60 ml.) and water (40 ml.),

(ii) Reagent B: orcinol (0.4 g.) and water (25 ml.).

Reagent A (7.5 ml.) was added to Reagent B (1 ml.), the resulting solution was cooled to 5° and used soon after preparation. A series of test tubes (150 x 25 mm.) were prepared as follows:

Control; 1 ml. of water

Standards; 1 ml. of mannose solution (100 µg./ml.)
1 ml. of mannose solution (200 µg./ml.)
1 ml. of mannose solution (300 µg./ml.)
1 ml. of mannose solution (400 µg./ml.)
1 ml. of mannose solution (500 µg./ml.)

Test solutions; 1 ml. aliquots from each fraction obtained from the Sephadex columns.

To each of the above test tubes was added 8.5 ml. of the orcinol-sulfuric acid solution. The test tubes (150 x 25 mm.) had a small constriction near the top so that smaller test tubes (13 x 75 mm.) filled with cold water could be placed in the neck to act as condensers. The large test tubes were then placed in water at 80° for 15 minutes, cooled, and the optical density of each solution at 505 mµ read in a Unicam SP 600 Spectrophotometer. A standard curve (Figure 11) was constructed and the mannose content of the test solutions determined from it by reading the mannose concentrations corresponding to the optical densities of the solutions.

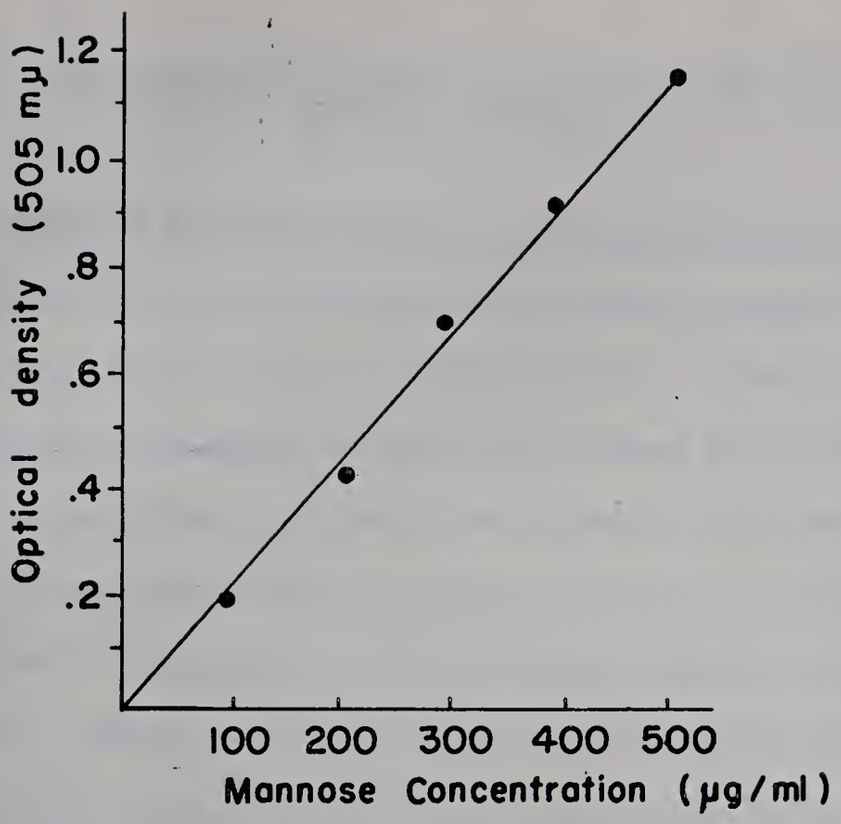


Figure II. Mannose Determination Standard Curve

## STUDIES OF THE EFFECTS OF COMMERCIAL AVIDIN ON THE ALA SYNTHETASE OF CHICKEN ERYTHROCYTES

### (i) Preparation and Incubation of Erythrocytes for Porphyrin Synthesis [33]

Chicken blood was obtained from a Kosher slaughter house where chickens were killed by cutting their throats. The blood was collected in a polythene container containing one volume of 0.2 M sodium citrate for each nine volumes of blood. The citrated blood was centrifuged at 1000 g for ten minutes and the plasma, platelet and leucocyte layers were removed by aspiration. The red blood cells were washed twice with 0.25 M cold sucrose and the packed cells suspended in an equal volume of a solution containing 0.05 M phosphate buffer, pH 7.4, 0.25 M sucrose, and 0.4 mg. of streptomycin per ml. For each experiment aliquots (5 ml.) of a suspension prepared in this manner were diluted with 0.6 ml. of a solution containing 0.07 M glycine and 0.01 M $\alpha$-ketoglutarate and added to test tubes (150 x 25 mm.). Commercial avidin (3.6 mg.) was added to half of the test tubes. These constituted the test samples while the remainder constituted the control samples. Three control and three test samples were incubated at 38° for 11 hours on a shaker. Similar sets of control and test samples were incubated for periods of 0, 16, and 21 hours and the amount of protoporphyrin formed then estimated as described below. Those tubes which were not analyzed immediately after incubation were preserved at -15° for one to several days prior to analysis. [33]

### (ii) Extraction and Estimation of Porphyrins

The method developed by Granick [33] was used. The erythrocyte sus-

pensions which were incubated for varying lengths of time were extracted three times, each time with 20 ml. portions of ethyl acetate-acetic acid (3:1v/v) followed by centrifugation. The combined extract was neutralized with a saturated solution of sodium acetate and washed with water. The porphyrins were extracted from the ethyl acetate layer using three portions of 2.5 N hydrochloric acid (3.3 ml.). The combined acid extracts were placed in a small separating funnel containing ether (6 ml.). The aqueous phase was brought to pH 4 by the addition of a saturated solution of sodium acetate whereupon the porphyrins passed into the ether phase. The aqueous phase was discarded and the ether phase washed with water. Coproporphyrin was selectively extracted from the porphyrin mixture with two portions of 0.05 N hydrochloric acid (2.5 ml.). The extraction of protoporphyrin from the ether layer was accomplished with two portions of 2.5 N hydrochloric acid (2.5 ml.). This acid extract was placed in a volumetric flask, diluted with absolute alcohol to 10 ml. and the optical density read at 409 mμ in the Bausch and Lomb Ultraviolet Spectrophotometer. The amount of protoporphyrin present was determined from the value of the Emax $(2.6 \times 10^5)$[33] at 409 mμ. The values plotted in Figure 9 are the average of triplicate determinations which are given below in Table 9.

TABLE 9

Protoporphyrin Formed in Chicken Erythrocytes (in mμ moles)

| Hrs. of Incubation | Control Samples | Test Samples (Commercial Avidin Added) |
|---|---|---|
| 0 | .04, .07, .04 | .06, .05, .05 |
| 11 | 2.31, 2.27, 2.77 | 2.54, 2.62, 2.65 |
| 16 | 3.54, 3.77, 3.65 | 3.50, 3.65, 3.46 |
| 21 | 4.92, 4.72, 4.81 | 4.73, 4.54, 4.73 |

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